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Practitioner's Docket No. 6596

CHAPTER II

Preliminary Classification:

Proposed Class:

Subclass:

NOTE: "All applicants are requested to include a preliminary classification on newly filed patent applications. The preliminary classification, preferably class and subclass designations, should be identified in the upper right-hand corner of the letter of transmittal accompanying the application papers, for example 'Proposed Class 2, subclass 129.' " M.P.E.P., § 601, 7th ed

TRANSMITTAL LETTER TO THE UNITED STATES ELECTED OFFICE (EO/US)

(ENTRY INTO U.S. NATIONAL PHASE UNDER CHAPTER II)

PCT/IT00/00321	28 July 2000	6 August 1999
INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED

NON-HUMAN TRANSGENIC ANIMALS FOR THE STUDY OF NEURODEGENERATIVE SYNDROMES

TITLE OF INVENTION

APPLICANT(S)

CATTANEO, Antonino; CAPSONI, Simona and RUBERTI, Francesca

Box PCT
Assistant Commissioner for Patents
Washington D.C. 20231

ATTENTION: EO/US

CERTIFICATION UNDER 37 C.F.R. § 1.10*

*(Express Mail label number is **mandatory**.)*
(Express Mail certification is optional.)

I hereby certify that this Transmittal Letter and the papers indicated as being transmitted therewith is being deposited with the United States Postal Service on this date 5 February 2002, in an envelope as "Express Mail Post Office to Addressee" Mailing Label Number EL737903405US, addressed to the Assistant Commissioner for Patents, 2900 Crystal Drive, Arlington, VA 22202.

Sarah E. Kennedy

(type or print name of person mailing paper)

Signature of person mailing paper

***WARNING:** Certificate of mailing (first class) or facsimile transmission procedures of 37 C.F.R. § 1.8 cannot be used to obtain a date of mailing or transmission for this correspondence.*

***WARNING:** Each paper or fee filed by "Express Mail" **must** have the number of the "Express Mail" mailing label placed thereon prior to mailing. 37 C.F.R. § 1.10(b).*
*"Since the filing of correspondence under § 1.10 without the Express Mail mailing label thereon is an oversight that can be avoided by the exercise of reasonable care, requests for waiver of this requirement will **not** be granted on petition." Notice of Oct. 24, 1996, 60 Fed. Reg. 56,439, at 56,442.*

NOTE. To avoid abandonment of the application, the applicant shall furnish to the USPTO, not later than 20 months from the priority date: (1) a copy of the international application, unless it has been previously communicated by the International Bureau or unless it was originally filed in the USPTO; and (2) the basic national fee (see 37 C.F.R. § 1.492(a)). The 30-month time limit may not be extended. 37 C.F.R. § 1.495.

***WARNING:** Where the items are those which can be submitted to complete the entry of the international application into the national phase are subsequent to 30 months from the priority date the application is still considered to be in the international state and if mailing procedures are utilized to obtain a date the express mail procedure of 37 C.F.R. § 1.10 must be used (since international application papers are not covered by an ordinary certificate of mailing—See 37 C.F.R. § 1.8.*

NOTE: Documents and fees must be clearly identified as a submission to enter the national state under 35 U.S.C. § 371 otherwise the submission will be considered as being made under 35 U.S.C. § 111. 37 C.F.R. § 1.494(f).

I. Applicant herewith submits to the United States Elected Office (EO/US) the following items under 35 U.S.C. § 371:

a. ☒ This express request to immediately begin national examination procedures (35 U.S.C. § 371(f)).

b. ☐ The U.S. National Fee (35 U.S.C. § 371(c)(1)) and other fees (37 C.F.R. § 1.492) as indicated below:

2. Fees

CLAIMS FEE	(1) FOR	(2) NUMBER FILED	(3) NUMBER EXTRA	(4) RATE	(5) CALCULATIONS
☒*	TOTAL CLAIMS	37 -20=	17	× $18.00=	$306.00
	INDEPENDENT CLAIMS	5 -3=	2	× $80.00=	$168.00
	MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $270.00	
BASIC FEE**	☐ U.S. PTO WAS INTERNATIONAL PRELIMINARY EXAMINATION AUTHORITY Where an International preliminary examination fee as set forth in § 1.482 has been paid on the international application to the U.S. PTO: ☐ and the international preliminary examination report states that the criteria of novelty, inventive step (non-obviousness) and industrial activity, as defined in PCT Article 33(1) to (4) have been satisfied for all the claims presented in the application entering the national stage (37 C.F.R. § 1.492(a)(4)) ..... $100.00 ☐ and the above requirements are not met (37 C.F.R. § 1.492(a)(1)) ..... $690.00 ☒ U.S. PTO WAS NOT INTERNATIONAL PRELIMINARY EXAMINATION AUTHORITY Where no international preliminary examination fee as set forth in § 1.482 has been paid to the U.S. PTO, and payment of an international search fee as set forth in § 1.445(a)(2) to the U.S. PTO: ☐ has been paid (37 C.F.R. § 1.492(a)(2)) ..... $710.00 ☐ has not been paid (37 C.F.R. § 1.492(a)(3)) ..... $1000.00 ☒ where a search report on the international application has been prepared by the European Patent Office or the Japanese Patent Office (37 C.F.R. § 1.492(a)(5)) $860.00				$890.00
	Total of above Calculations				$1,364.00
SMALL ENTITY	Reduction by 1/2 for filing by small entity, if applicable. Affidavit must be filed also. (note 37 C.F.R. § 1.9, 1.27, 1.28)				-
	Subtotal				$1,364.00
	Total National Fee				$ 1,364.00
	Fee for recording the enclosed assignment document $40.00 (37 C.F.R. § 1.21(h)). (See Item 13 below). See attached "ASSIGNMENT COVER SHEET".				
TOTAL	Total Fees enclosed				$1,364.00

*See attached Preliminary Amendment Reducing the Number of Claims.

☒ Attached is a ☒ check ☐ money order in the amount of $ 1,364.00

☒ Authorization is hereby made to charge the amount of $ _____

☒ to Deposit Account No. 19-0079

☐ to Credit card as shown on the attached credit card information authorization form PTO-2038.

WARNING: *Credit card information should* ***not*** *be included on this form as it may become public.*

☒ Charge any additional fees required by this paper or credit any overpayment in the manner authorized above.

A duplicate of this paper is attached.

WARNING: *"To avoid abandonment of the application the applicant shall furnish to the United States Patent and Trademark Office not later than the expiration of 30 months from the priority date: * * * (2) the basic national fee (see § 1.492(a)). The 30-month time limit may not be extended." 37 C.F.R. § 1.495(b).*

WARNING: *If the translation of the international application and/or the oath or declaration have not been submitted by the applicant within thirty (30) months from the priority date, such requirements may be met within a time period set by the Office. 37 C.F.R. § 1 495(b)(2). The payment of the surcharge set forth in § 1.492(e) is required as a condition for accepting the oath or declaration later than thirty (30) months after the priority date. The payment of the processing fee set forth in § 1.492(f) is required for acceptance of an English translation later than thirty (30) months after the priority date. Failure to comply with these requirements will result in abandonment of the application. The provisions of § 1.136 apply to the period which is set. Notice of Jan. 3, 1993, 1147 O.G. 29 to 40.*

3. ☒ A copy of the International application as filed (35 U.S.C. § 371(c)(2)):

NOTE: Section 1.495 (b) was amended to require that the basic national fee and a copy of the international application must be filed with the Office by 30 months from the priority date to avoid abandonment "The International Bureau normally provides the copy of the international application to the Office in accordance with PCT Article 20. At the same time, the International Bureau notifies applicant of the communication to the Office. In accordance with PCT Rule 47.1, that notice shall be accepted by all designated offices as conclusive evidence that the communication has duly taken place. Thus, if the applicant desires to enter the national stage, the applicant normally need only check to be sure the notice from the International Bureau has been received and then pay the basic national fee by 30 months from the priority date." Notice of Jan. 7, 1993, 1147 O.G. 29 to 40, at 35-36. See item 14c below.

a. ☐ is transmitted herewith.

b. ☐ is not required, as the application was filed with the United States Receiving Office.

c. ☒ has been transmitted

i. ☒ by the International Bureau.
Date of mailing of the application (from form PCT/1B/308):
15 February 2001

ii. ☐ by applicant on _____. *(Date)*

4. ☒ A translation of the International application into the English language (35 U.S.C. § 371(c)(2)):

a. ☐ is transmitted herewith.

b. ☒ is not required as the application was filed in English.

c. ☐ was previously transmitted by applicant on _____. *(Date)*

d. ☐ will follow.

5. ☒ Amendments to the claims of the International application under PCT Article 19 (35 U.S.C. § 371(c)(3)):

NOTE. The Notice of January 7, 1993 points out that 37 C.F.R § 1.495(a) was amended to clarify the existing and continuing practice that PCT Article 19 amendments must be submitted by 30 months from the priority date and this deadline may not be extended. The Notice further advises that: "The failure to do so will not result in loss of the subject matter of the PCT Article 19 amendments. Applicant may submit that subject matter in a preliminary amendment filed under section 1.121. In many cases, filing an amendment under section 1.121 is preferable since grammatical or idiomatic errors may be corrected." 1147 O.G. 29-40, at 36.

a. ☐ are transmitted herewith.

b. ☐ have been transmitted

i. ☐ by the International Bureau.
Date of mailing of the amendment (from form PCT/1B/308):
_____.

ii. ☐ by applicant on _____. *(Date)*

c. ☒ have not been transmitted as

i. ☒ applicant chose not to make amendments under PCT Article 19.
Date of mailing of Search Report (from form PCT/ISA/210.):
8 January 2002

ii. ☐ the time limit for the submission of amendments has not yet expired. The amendments or a statement that amendments have not been made will be transmitted before the expiration of the time limit under PCT Rule 46.1.

6. ☒ A translation of the amendments to the claims under PCT Article 19 (38 U.S.C. § 371(c)(3)):

a. ☐ is transmitted herewith.

b. ☐ is not required as the amendments were made in the English language.

c. ☒ has not been transmitted for reasons indicated at point 5(c) above.

7. ☐ A copy of the international examination report (PCT/IPEA/409)

☐ is transmitted herewith.

☐ is not required as the application was filed with the United States Receiving Office.

8. ☐ Annex(es) to the international preliminary examination report

a. ☐ is/are transmitted herewith.

b. ☐ is/are not required as the application was filed with the United States Receiving Office.

9. ☐ A translation of the annexes to the international preliminary examination report

a. ☐ is transmitted herewith.

b. ☐ is not required as the annexes are in the English language.

10. ☒ An oath or declaration of the inventor (35 U.S.C. § 371(c)(4)) complying with 35 U.S.C. § 115

a. ☐ was previously submitted by applicant on _____. Date

b. ☐ is submitted herewith, and such oath or declaration

i. ☐ is attached to the application.

ii. ☐ identifies the application and any amendments under PCT Article 19 that were transmitted as stated in points 3(b) or 3(c) and 5(b); and states that they were reviewed by the inventor as required by 37 C.F.R. § 1.70.

c. ☒ will follow.

II. Other document(s) or information included:

11. ☒ An International Search Report (PCT/ISA/210) or Declaration under PCT Article 17(2)(a):

a. ☒ is transmitted herewith.

b. ☐ has been transmitted by the International Bureau. Date of mailing (from form PCT/IB/308): _____.

c. ☐ is not required, as the application was searched by the United States International Searching Authority.

d. ☐ will be transmitted promptly upon request.

e. ☐ has been submitted by applicant on _____. Date

12. ☒ An Information Disclosure Statement under 37 C.F.R. §§ 1.97 and 1.98:

a. ☒ is transmitted herewith.

Also transmitted herewith is/are:

☒ Form PTO-1449 (PTO/SB/08A and 08B).

☒ Copies of citations listed.

b. ☐ will be transmitted within THREE MONTHS of the date of submission of requirements under 35 U.S.C. § 371(c).

c. ☐ was previously submitted by applicant on _____. Date

13. ☐ An assignment document is transmitted herewith for recording.

A separate ☐ "COVER SHEET FOR ASSIGNMENT (DOCUMENT) ACCOMPANYING NEW PATENT APPLICATION" or ☐ FORM PTO 1595 is also attached.

_____

_____

_____

14. ☒ Additional documents:

a. ☒ Copy of request (PCT/RO/101)

b. ☒ International Publication No. WO 01/10203

i. ☒ Specification, claims and drawing

ii. ☐ Front page only

c. ☒ Preliminary amendment (37 C.F.R. § 1.121)

d. ☒ Other

PCT/IB/304; PCT/IB/332; PCT/IPEA/402

15. ☒ The above checked items are being transmitted

a. ☒ before 30 months from any claimed priority date.

b. ☐ after 30 months.

16. ☐ Certain requirements under 35 U.S.C. § 371 were previously submitted by the applicant on _____, namely:

AUTHORIZATION TO CHARGE ADDITIONAL FEES

WARNING: *Accurately count claims, especially multiple dependant claims, to avoid unexpected high charges if extra claims are authorized.*

NOTE: "A written request may be submitted in an application that is an authorization to treat any concurrent or future reply, requiring a petition for an extension of time under this paragraph for its timely submission, as incorporating a petition for extension of time for the appropriate length of time. An authorization to charge all required fees, fees under § 1.17, or all required extension of time fees will be treated as a constructive petition for an extension of time in any concurrent or future reply requiring a petition for an extension of time under this paragraph for its timely submission. Submission of the fee set forth in § 1.17(a) will also be treated as a constructive petition for an extension of time in any concurrent reply requiring a petition for an extension of time under this paragraph for its timely submission." 37 C.F.R. § 1.136(a)(3).

NOTE: "Amounts of twenty-five dollars or less will not be returned unless specifically requested within a reasonable time, nor will the payer be notified of such amounts; amounts over twenty-five dollars may be returned by check or, if requested, by credit to a deposit account." 37 C.F.R. § 1.26(a).

☒ Please charge, in the manner authorized above, the following additional fees that may be required by this paper and during the entire pendency of this application:

☒ 37 C.F.R. § 1.492(a)(1), (2), (3), and (4) (filing fees)

WARNING: *Because failure to pay the national fee within 30 months without extension (37 C.F.R. § 1.495(b)(2)) results in abandonment of the application, it would be best to always check the above box.*

☒ 37 C.F.R. § 1.492(b), (c) and (d) (presentation of extra claims)

NOTE: Because additional fees for excess or multiple dependent claims not paid on filing or on later presentation must only be paid or these claims cancelled by amendment prior to the expiration of the time period set for response by the PTO in any notice of fee deficiency (37 C.F.R. § 1.492(d)), it might be best not to authorize the PTO to charge additional claim fees, except possible when dealing with amendments after final action.

☒ 37 C.F.R. § 1.17 (application processing fees)

☒ 37 C.F.R. § 1.17(a)(1)–(5) (extension fees pursuant to § 1.136(a).

☐ 37 C.F.R. § 1.18 (issue fee at or before mailing of Notice of Allowance, pursuant to 37 C.F.R. § 1.311(b))

NOTE: Where an authorization to charge the issue fee to a deposit account has been filed before the mailing of a Notice of Allowance, the issue fee will be automatically charged to the deposit account at the time of mailing the notice of allowance. 37 C.F.R. § 1.311(b).

NOTE: 37 C.F.R. § 1.28(b) requires "Notification of any change in loss of entitlement to small entity status must be filed in the application . . . prior to paying, or at the time of paying . . . issue fee." From the wording of 37 C.F.R. § 1.28(b): (a) notification of change of status must be made even if the fee is paid as "other than a small entity" and (b) no notification is required if the change is to another small entity.

☐ 37 C.F.R. § 1.492(e) and (f) (surcharge fees for filing the declaration and/or filing an English translation of an International Application later than 30 months after the priority date).

SIGNATURE OF PRACTITIONER

Reg. No.: 35,985

Arlene J. Powers

(type or print name of practitioner)

Tel. No.: (617) 426-9180 ext. 110

225 Franklin Street, Suite 3300

P.O. Address

Customer No.:

Boston, Massachusetts 02110

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT:	Cattaneo et al.	**GROUP:**	Unknown
SERIAL NO:	Unknown	**EXAMINER:**	Unknown
FILED:	Herewith		
FOR:	NON-HUMAN TRANSGENIC ANIMALS FOR THE STUDY OF NEURODEGENERATIVE SYNDROMES		

Assistant Commissioner of Patents
Washington, D.C. 20231
Sir:

PRELIMINARY AMENDMENT

Preliminary to examination, please amend the above-identified application as follows:

IN THE ABSTRACT:

An abstract is attached hereto on a separate sheet.

IN THE CLAIMS:

Please amend claim 10 as follows:

10. (Amended) A non-human transgenic animal according to claim 1 being transgenic for an anti-NGF (Nerve Growth Factor) antibody or fragment thereof.

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited on the date shown below in an envelope as "Express Mail Post Office to Addressee" Mailing Label Number EL737903405US addressed to the: Commissioner of Patents and Trademarks, 2900 Crystal Drive, Arlington, VA 22202..

Sarah Kennedy

Date. 2/5/02

Please amend claim 17 as follows:

17. (Amended) A non-human transgenic animal according to claim 1, wherein the animal is a mammalian.

Please amend claim 21 as follows:

21. (Amended) Cells derivable from the non-human transgenic animal according to claim 1 and secreting the transgene antibody.

REMARKS

The present preliminary amendment is submitted in order to correct the improper multiple dependency of claims as originally filed.

Examination on the merits is respectfully requested.

Respectfully submitted,

Arlene J. Powers
Registration No. 35,985
Samuels, Gauthier & Stevens
225 Franklin Street, Suite 3300
Boston, Massachusetts 02110
Telephone: (617) 426-9180
Extension 110

ABSTRACT

A non-human transgenic animal that is transgenic for an antibody or fragments thereof and having a phenotype reminiscent of human pathology. The human pathology includes neurodegenerative syndromes, muscular atrophy/dystrophy and immune disorders. The animals may be used in a method for early diagnosis of neurodegenerative diseases. The method includes monitoring the occurrence of the tau hyperphosphorylation and/or amyloid deposition in the back or lower limb skeletal muscle sample of a subject. Cells are derivable from the non-human transgenic animal and secreting the transgenic antibody. The cells are used for the selection of molecules pharmacologically effective in neurodegenerative and/or muscular pathologies and/or immune disorders. A non-human transgenic animal may be prepared by providing a first non-human transgenic parent animal for the light chain of an antibody and a second non-human transgenic parent animal for the heavy chain of the same antibody, breeding the two transgenic parent animals and selecting the progeny expressing both the light and the heavy chain.

IN THE CLAIMS:

Claim 10 has been amended as follows:

10. (Amended) A non-human transgenic animal according to [any of previous claims] claim 1 being transgenic for an anti-NGF (Nerve Growth Factor) antibody or fragment thereof.

Claim 17 has been amended as follows:

17. (Amended) A non-human transgenic animal according to [any of previous claims] claim 1, wherein the animal is a mammalian.

Claim 21 has been amended as follows:

21. (Amended) Cells derivable from the non-human transgenic animal according to [any of claims 1-19] claim 1 and secreting the transgene antibody.

PROCESS FOR THE PRODUCTION OF TUNGSTEN-COPPER COMPOSITE SINTERABLE POWDERS

SPECIFICATION

The present invention relates to a process for the production of sinterable tungsten-copper composite powders. More particularly the invention relates to a method for the production of a composite powder consisting of finely interspersed tungsten and copper, which powder can be directly pressed and sintered to provide products having density values near to theoretical ones and showing high electrical and thermal conductivity.

Tungsten-copper composite materials are used for the production of heat exchangers for electrical devices and for the production of electrodes and power electrical contacts. Since alloying does not occur between tungsten and copper, various methods have been developed to combine these metals in order to obtain products wherein the low coefficient of thermal expansion and the advantageous mechanical properties of tungsten are coupled to the high electrical and thermal conductivity of copper.

The method most widely used to this aim (known as infiltration technique) comprises: i) sintering a tungsten metal powder at such a temperature to obtain a porous tungsten structure; ii) infiltrating said structure with molten copper, the pores of the structure being filled by the liquid metal (see, for example, Randall M. German, "Sintering Theory and Practice", pages 385-389, John Wiley & Sons, Inc., New York (1996).

The amount of copper which can be incorporated in sintered tungsten depends, however, on the porosity of the latter, which in turn depends on the starting grain size of tungsten powder and on the sintering conditions. Furthermore, in order to be filled by molten copper, the pores must be open or it is necessary that the fraction of closed pores in the starting sintered tungsten be minimal. Where there are closed pores, through which copper cannot flow thus filing them, fragile products are obtained. Thus the need to minimize the presence of closed pores makes the first step during the production process a

critical one, and limits the range of the obtainable tungsten-copper compositions. In addition, although tungsten porous structures having shapes similar to those of end products can be produced, following the infiltration process further machining of products is necessary in order to remove the excess of copper, flowed out of the pores of the tungsten structure, and in order to obtain again the desired shapes. Also in view of this aspect, the use of the infiltration process is convenient, from an economic standpoint, only for producing products having quite simple shapes.

The latter limitation, together with the need to carry out two high temperature treatments (the tungsten sintering and the following infiltration of the resulting porous structure by molten copper), as well as the machining needed for re-obtaining the product with desired size, reduce the commercial applicability of the infiltration process and result in a need for a direct sintering of mixtures of tungsten and copper powders.

However, the conventional powder metallurgy methods for the production of components by mixing, pressing and sintering mixtures of tungsten and copper as elemental powders did not prove commercially convenient, particularly where the copper content is low (from 5 to 20% by weight). In fact the high residual porosity in the products obtained by using the pressing and sintering process, which impairs not only mechanical strength properties but also and particularly the electrical and thermal conductivity, results in the need for further re-pressing and re-sintering processes. (D. L. Houck, L.P. Dorfman, M. Paliwal, "Tungsten/Copper Composites for Heat Sinks and Electrical Contacts", in Proceedings of 7th International Tungsten Symposium, pages 390-409, 24-27 September 1996, Goslar, Germany).

Another set of methods for obtaining tungsten-copper composite powders include the steps of mixing/grinding and the following co-reduction in hydrogen atmosphere of copper oxide and tungsten oxide powders. The thus obtained metal particles are in more intimate contact than that obtainable by using only mechanical grinding of copper and tungsten metals and the resulting tungsten-copper powder can be directly pressed and sintered to density values exceeding 95 % of the theoretical ones.

Also compounds as copper tungstate ($CuWO_4$), wherein copper and tungsten are mixed at the atomic level, can be reduced to obtain tungsten-copper composite powders having good sintering properties. According to conventional methods, copper tungstate is produced by reacting in the solid phase CuO with WO_3; in order to obtain an intimate contact between the two oxide phases, however, it is necessary to grind for a long time the CuO-WO_3 mixture by means of balls made of hard metal or ceramic material, thus resulting in a potentially contaminated mixture. Furthermore high temperatures and long calcining times impair the process for producing W-Cu powders from an economic standpoint, although metallic powders obtained from tungstate have good interspersion and sintering properties.

In order to reduce temperatures and calcining times, U.S. patent No. 5468457 suggests to use as precursors, instead of conventional oxides, hydrated oxides, i.e. copper hydroxide, $Cu(OH)_2$ (i.e. $CuO.H_2O$) and tungstic acid, H_2WO_4 (i.e. $WO_3.H_2O$). The heat treatment of such a mixture of hydrated oxides results in water development with formation of CuO and WO_3 with high surface area, which assures the advantage of higher reactivity in the following step at higher temperatures (600-800°C).

Furthermore, with reference to the production of the $CuWO_4$ mixed oxide, U.S. patent No. 54670549 discloses an alternative route with respect to the above mentioned one, which includes the use of ammonium tungstate (both meta-tungstate, AMT, and para-tungstate, APT) as tungsten precursors, while both CuO and Cu_2O can be used as copper precursors. Tungsten oxide (WO_3), obtained from the ammonium tungstate decomposition at temperatures higher than 250°C, shows a high reactivity and therefore there is no more the need for the starting grinding step to promote the contacting and the following reaction between the oxide precursors. As the Cu/W ratio in $CuWO_4$ is fixed (25.7% by weight in the final W-Cu powder), in order to obtain metal powders with different copper content it is necessary to modify the amount of copper oxides or to add WO_3 to the tungstate.

In all the above described processes the production of composite tungsten-copper powders having suitable interspersion of the two metallic

phases requires carrying out of at least two of the following preparative steps :

i) mixing and possible dry milling of copper oxides or hydroxides with tungsten oxides, hydrated oxides or other tungsten compounds, such as AMT or APT;

ii) solid state reacting at temperatures between 600 and 800°C;

iii) treating in hydrogen atmosphere for a sufficient time to achieve a complete reduction of oxide type compounds.

An usual drawback of such methods (in addition, of course, to the need for high temperatures) is due to the fact that they require a careful control of operating conditions in order to obtain a composite metallic powder having a suitable grain size. Furthermore, the thus obtained tungsten-copper powders tend to form aggregates, which reduces their usefulness for producing small components as required in electronic industry.

As an alternative to methods wherein tungsten and copper precursors are dry mixed/grounded, U.S. patent No. 5439638 suggests a process for the production of tungsten-copper composite powders having copper contents in the range between 5 and 60% by weight, wherein the starting ingredients are wet mixed. More particularly the process uses starting powders comprising elemental tungsten, cuprous oxide and, optionally, cobalt powder at level less than 0.5% by weight. The powders are first interspersed in an aqueous medium, then the liquid is removed by spray-driyng; in a such way a flowable powder comprising spherical aggregates is obtained. Ultimately cuprous oxide (Cu_2O) is reduced in hydrogen atmosphere at 700-730°C to produce a tungsten-copper sinterable powder, in the form of spherical aggregates too.

A technique, partially similar to the various methods mentioned above, including a step for a dry or aqueous phase powder mixing, followed by high temperature reduction, is described in European Patent Publication EP-A-0806489. The latter teaches that W/Cu products, having density values above 97% of theoretical, are directly obtained by using starting mixtures containing copper and a transition metal (as W or Mo), provided that the mixture also contains chemically bonded oxygen, for example in the form of copper oxide, in such amounts to improve the sinterability thereof. The described procedure

preferably includes mechanical mixing of elemental tungsten and cuprous oxide powders, which, following their pressing and high temperature treating in hydrogen atmosphere, results in the formation of a sintered product.

Besides by reduction in hydrogen atmosphere at high temperatures, metallic powders can be produced by liquid phase reduction using an alcohol solvent as reducing agent.

For example, according to the method suggested in European Patent EP-B-0113281 monometallic powders (of gold, palladium, platinum, iridium, osmium, copper, silver, nickel, cobalt, lead or cadmium) can be produced by reduction from a precursor by using an organic liquid phase made up of one or a mixture of polyols. More particularly a compound of the desired metal selected from oxides, hydroxides and metal salts is reduced by the organic liquid phase by heating the mixture to a temperature of at least 85°C. Owing to the reduction, the metal is separated in the form of high purity powder.

Such a patent, however, does not disclose nor suggest the production of mixed metallic powders, for example by co-reducing two different metal precursors. Furthermore the process suggested requires the use of temperatures which are always higher than 85°C (preferably in the range 100-350°C).

A further development of the above described "polyol method" is reported in U.S. Patent No. 5759230, which is relevant to the production of metallic films and powders with sizes in the nanometric range comprising one or more metal elements, sometimes alloyed. In this case, as above, the reducing agent is formed by an alcohol phase, typically a polyol, wherein one or more precursors are suspended (typically in the form of metal salt, hydrated salt or oxy-anion). The method is suggested for the production of nanostructured films and powders of one or more metals selected from Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Nb, Mo, Ru, Rh, Pd, Ag, In, Sn, Ta, W, Re, Os, Ir, Pt and Au. According to a specific embodiment the method provides powders consisting of refractory metals (W, Ti, Mo, Re, Ta) or their alloys produced from salts or acids which contain said metals in the corresponding oxy-anions.

Although the general method is described with reference to several

single metals and alloys or metal composites, its application does not allow the co-reduction of W and Cu precursors in order to produce sinterable tungsten-copper composite powders and the specific examples in fact do not disclose such a combination.

Accordingly it is an object of the present invention to provide a procedure for the production of directly sinterable tungsten-copper composite metal powders based on a liquid phase reduction of precursors thereof, which procedure takes advantage from the polyol method, in that it does no require high temperature treatments and affords to obtain in a single step both the mixing and the reduction of the two powders and, at the same time, is suitable specifically for the two mentioned metals.

According to the invention it has been found that it is possible to produce tungsten-copper composite powders suitable to be used directly for the production of sintered products, by using a reduction process in a liquid organic phase consisting of one or a mixture of polyols wherein copper is added as precursor compound whereas tungsten is added as metal. In fact it has been found that the presence of elemental tungsten is necessary in order to achieve the reduction of the copper precursor at reasonably low temperature and short time, as tungsten itself takes part in the copper compound reduction, thus allowing the reduction reaction to occur at lower temperatures. In fact it has been found that in the presence of elemental tungsten the organic phase reaction can be carried out below the lowest temperature values known in the art (85°C).

Therefore the present invention specifically provides a method for the production of tungsten-copper composite powders suitable to be pressed and sintered and having a copper content from 5 to 35% by weight, the method comprising the following steps:

a) suspending an elemental tungsten powder in one or a mixture of liquid polyols;

b) adding to the thus obtained suspension a copper precursor and, optionally, minor amounts of other metal precursors;

c) heating the resulting suspension to a temperature of at least 60°C

and keeping it under stirring at such a temperature for a sufficient time to allow the reduction of said copper precursor and of said other optional metal precursors ;

d) separating the solid phase from the suspension obtained from the preceding step and washing it with an organic solvent, thus obtaining a powder consisting of a tungsten-copper mixture optionally containing other metal elements, wherein all the metals are highly interspersed.

Preferably the organic phase wherein the oxidation-reduction reaction and concurrent interspersion of the produced copper and the starting tungsten occur, consists of ethylene glycol, pure or in admixture with other polyols, as for example diethylene glycol. The starting elemental tungsten powder can be any commercially available powder having an average grain size preferably in the range from 0.5 to 6 μm. The copper compound can be either soluble in the polyol, as is the case, for example, of copper (II) acetate monohydrate ($Cu(CH_3COO)_2.H_2O$) or insoluble in the polyol, as is the case of cupric and cuprous oxides (CuO and Cu_2O respectively).

The method suggested in accordance with the present invention allows the preparation of tungsten-copper composite powders having a broad composition range since, in order to obtain the desired proportions in the final composite powder, it is only required to modify the starting relative amounts of tungsten and copper compound present in the organic phase suspension/solution. In this connection it is to be noted, besides the fact that copper is added to the reaction mixture in the form of a precursor compound, that the starting elemental tungsten, being active in the copper reduction, undergoes a partial solubilization as tungstate and therefore its concentration in the final metallic product is reduced. Suitable starting amounts of elemental tungsten and copper compound to be used for producing a composite powder having desired W/Cu ratios can be easily established by those skilled in the art on the basis of reaction yields for a few exemplary experiments, as illustrated in the following examples.

As above specified, the temperature of the organic phase, wherein the copper compound reduction occurs, is at least 60°C. The time during which

the reduction process is achieved depends on the selected temperature; for example, at 70°C the reduction is completed over 4-6 hours, whereas at the boiling temperature of the polyol employed (preferably ethylene glycol, T_{eb} = 198°C) the reaction is completed over 5-15 minutes.

The composite powders obtained by using the method of the present invention can be stored for a long time wet with same organic solvent, thus avoiding any risk of spontaneous ignition of the dry powders. Possible residues of organic phases which can be present after the composite tungsten-copper powder washings are removed during the sintering cycle.

Furthermore it is possible to control the microstructure of the final powder by modifying: *i)* the grain size of the tungsten starting powder, *ii)* the composition of the organic phase employed, *iii)* the copper precursor and concentration thereof, *iv)* the reaction temperature and time.

It is also possible to obtain composite tungsten-copper powders containing suitable additives for reducing sintering temperatures or times and/or improving technological and utilization properties of the product obtained. For example by adding to the tungsten suspension (in addition to the copper precursor) a suitable amount of a cobalt (II) compound, as, for example, cobalt (II) acetate tetrahydrate, at the reaction temperature cobalt metal is formed, which, in small amounts, allows to lower the W-Cu composite sintering temperature and/or time (S. K. Joo, S. W. Lee, T. H. Ihn, "Effect of Cobalt Addition on the Liquid Phase Sintering of W-Cu Prepared by the Fluidized Bed Reduction Method"; *Met. Mater. Trans.* Vol. 25A, pages 1575-1578 (1994)).

Specific embodiments of the method in accordance with the invention are described in the following by way of example only, and analytical data as well as results of experimental tests or the products obtained are reported.

EXAMPLE 1

670 g of tungsten powder having a grain size of 1 μm (H. C. Stark, grain size distribution 0.1-2.5 μm) was charged in a Pyrex glass reactor with 3.3 l of ethylene glycol (Carlo Erba). Thereafter 315 g of copper acetate monohydrate (corresponding to 100 g of copper metal) was added to the sus-

pension under stirring. The reaction was carried out at 70°C over 6 hours.

The reaction provided 670 g of product, weighed after the resulting composite powder had been separated, washed with acetone and air dried. The atomic absorption analysis showed a copper content of 15% by weight.

The reaction yield (87%) and the presence of tungsten ions in the organic phase suggest that tungsten takes an active part in the reduction reaction of the cuprous compound.

Pressing and sintering tests in hydrogen atmosphere as well as conductivity measurements have been carried out on the 85W-15Cu powder obtained and the results thereof are reported in the following table.

Pressing Load (ton/cm²)	Sintering Temperature	Relative density	Electrical conductivity (% IACS)
2.39	1350°C	97%	38

The above data point out that the obtained composite powder is suitable for pressing and sintering applications, relative density values above 96% being obtained. Correspondingly high values of electrical conductivity, required for the intended applications of these materials are obtained.

EXAMPLE 2

The same procedure of example 1 was repeated except that 397 g of tungsten was used, whereas the copper amount, in the form of copper acetate monohydrate, was kept constant (315 g). The reaction provided 400 g of product, weighed after the resulting composite powder had been separated, washed with acetone and air dried. The atomic absorption analysis showed a copper content of 25% by weight and the reaction yield was 81%.

The 75W-25Cu powder thus obtained has been pressed at 2.39 ton/cm^2 and sintered in hydrogen atmosphere at 1300°C, obtaining a density value of 98% of the theoretical one. The electrical conductivity of the sintered product was 46% IACS.

COMPARATIVE EXAMPLE 1

The same procedure of example 1 was repeated except that tungsten

was absent. After six hours at 70°C no formation of elemental copper was observed. This result proves that at the employed temperature the presence of tungsten is required in order to perform the reduction of the copper precursor to metallic copper.

COMPARATIVE EXAMPLE 2

5.30 g of sodium tungstate dihydrate ($Na_2WO_4.2H_2O$) were charged in a Pyrex glass flask with 60 ml of ethylene glycol. After two hours at the glycol boiling temperature (198°C) no chemical reaction was observed. This result proves that glycol is not suitable to carry out the reduction of sodium tungstate to elemental tungsten in the experienced reaction time, not even at its boiling temperature.

COMPARATIVE EXAMPLE 3

5.30 g of sodium tungstate dihydrate ($Na_2WO_4.2H_2O$) were charged in a Pyrex glass flask with 60 ml of diethylene glycol. After two hours at the glycol boiling temperature (245°C) no chemical reaction was observed. This result proves that not even by substituting the polyol it is possible to carry out the reduction of sodium tungstate to elemental tungsten powder.

COMPARATIVE EXAMPLE 4

4.00 g of tungstic acid (H_2WO_4) were charged in a Pyrex glass flask with 60 ml of ethylene glycol. After two hours at the glycol boiling temperature (198°C) no chemical reaction was observed. This result proves that under the indicated conditions neither tungstic acid, another potential tungsten precursor, can be reduced to tungsten metal by ethylene glycol.

COMPARATIVE EXAMPLE 5

5.30 g of sodium tungstate dihydrate and 1.60 g of copper acetate monohydrate were charged in a Pyrex glass flask with 60 ml of ethylene glycol. After two hours at the glycol boiling temperature (198°C) no chemical reaction was observed. The experiment proves that it is not possible to obtain W-Cu composite powders by co-reduction of copper and tungsten precursors by using ethylene glycol.

EXAMPLE 3

670 g of tungsten powder, having an average grain size of 4 μm, was

charged in a Pyrex glass reactor with 12 l of ethylene glycol (Carlo Erba). To the suspension under stirring 315 g of copper acetate monohydrate (corresponding to 100 g of copper metal) were then added. The reaction was carried out at the boiling temperature of ethylene glycol over 15 minutes.

The reaction provided 670 g of product, the yield being therefore 87%. Chemical analysis of the organic phase showed the presence of tungstate ions and the absence of cupric ions.

In order to establish the microstructure of the thus obtained W-Cu composite powder different analyses have been carried out. X-ray diffractometry (XRPD) showed that only W and Cu phases were present. Micrographs obtained by scanning electron microscopy (SEM) showed that, owing to the redox reaction they underwent, the tungsten grains had a modified morphology showing corrugated surfaces. Furthermore the obtained metallic copper nucleated on the tungsten particles in the form of small crystalline aggregates.

Energy dispersion microanalysis (EDS) showed a ratio of the Cu $K_{\alpha 1,2}$ to the W $L_{\alpha 1}$ peaks higher than that corresponding to powder mixtures containing 15% by weight of Cu and 85% by weight of W (85W-15Cu), confirming that the copper had nucleated on W particles with formation of a coating.

It is apparent, therefore, that in the process according to the invention the tungsten particles take an active part in the copper reduction, modifying their morphology with surface corrugations because of the oxidation to tungstate. Such a surface corrugation provides sites suitable for the heterogeneous nucleation of the elemental copper formed by reduction of the copper compound. Thus it is possible to achieve such an interspersion between the two metallic phases that results in the sinterability of the resulting W-Cu composite powder.

EXAMPLE 4

The procedure of example 3 was repeated except that the reaction was carried out at 110°C over two hours. After separation and washing with acetone of the obtained powder, microscopic analysis (SEM, EDS) showed that its microstructure and the interspersion of the two metals were the same

as in example 3.

The organic solution contained tungstate ions and the reaction yield was 87%.

EXAMPLE 5

The procedure of example 3 was repeated, using tungsten having an average grain size of 0.5 μm (Good Fellow). The reaction yield was 87% and again tungstate ions were present in the organic phase.

The structural characterization of the thus obtained 85W-15Cu composite powder was carried out by electron scanning microscopy and energy dispersion microanalysis and both these techniques showed excellent interspersion of the two metal phases.

EXAMPLE 6

The same procedure of example 3 was repeated except that 125 g of CuO (Aldrich) as copper precursor and tungsten having an average grain size of 0,5 μm (Good Fellow) were used. The reaction yield was 87% and tungstate ions were detected in the organic phase.

The thus obtained 85W-15Cu composite powder was showed to be completely similar to that obtained in example 5, indicating that also copper precursors insoluble in the reaction medium can be used for producing composite powders having highly interspersed metal phases.

EXAMPLE 7

670 g of tungsten powder having an average grain size of 1 μm (H. C. Stark) were charged in a Pyrex glass reactor with 3.3 l of ethylene glycol (Carlo Erba). Then to the suspension under stirring 315 g of copper acetate monohydrate were added (Carlo Erba). The reaction was carried out at the boiling temperature of glycol (198°C) over two hours.

The thus obtained tungsten-copper composite powder (670 g) was separated and washed with acetone. Pressing and sintering tests in hydrogen atmosphere as well as conductivity measurements were carried out and the results thereof are reported in the following table.

Pressing Load (ton/cm²)	Sintering temperature	Relative density	Electrical conductivity (% IACS)
0.95	1300°C	96%	40
	1350°C	97%	41
2.39	1300°C	96%	39
	1350°C	97%	41

EXAMPLE 8

The procedure of example 7 was repeated with the only difference that cobalt (II) acetate tetrahydrate was also added, in amount corresponding to a cobalt content in the final composite of 0.5 % by weight.

Following the reaction, the powder was pressed at 2.39 ton/cm^2 and sintered in hydrogen atmosphere at 1300°C, the density being 97% of the theoretical one. This result proves that the process of the invention allows concurrent adding of suitable sintering additives as elemental cobalt, herein formed by co-reduction of the corresponding salt in the organic phase.

By comparing the above indicated density value, relevant to a test with sintering temperature of 1300°C, with those indicated in the table of Example 7, it clearly appears that by adding cobalt in small amounts it is possible to achieve the same relative densities at lower sintering temperature.

EXAMPLE 9

670 g of tungsten powder, having an average grain size of 1 μm (H. C. Stark) was charged in a Pyrex glass reactor with 3.3 l of ethylene glycol (Carlo Erba). Then to the suspension under stirring 125 g of CuO (Aldrich) and 14 g of cobalt (II) acetate tetrahydrate (Carlo Erba) were added, the amounts being such as to have in the final product a copper content of 15% by weight and a cobalt content of 0.5 % by weight. The reaction was carried out at the boiling temperature of ethylene glycol (198°C) over two hours. The thus obtained tungsten-copper composite powder was separated and washed with acetone.

Thereafter the powder was pressed at 2.39 ton/cm^2 and sintered in

hydrogen atmosphere at 1350°C obtaining a density value of 98% of the theoretical one.

The obtained result proves that the method of the invention allows the production of W-Cu composite powders having high sinterability also by using a copper precursor which is insoluble in the organic phase wherein the reaction occurs.

As herein above pointed out, the method according to the invention allows the production of tungsten-copper composite powders suitable for the production of sintered products, having also complex shapes, without the need of using the conventional and more expensive infiltration method. With respect to the known methods for the production of tungsten-copper composite powders, according to which the mixing and reduction by hydrogen gas of oxide precursors are carried out at high temperature, the method of the invention has furthermore the advantage of carrying out both the copper reduction and the W and Cu interspersion in an organic liquid phase wherein tungsten powder is present, thus avoiding any preliminary process for the powder mixing and/or grinding.

Although the present invention has been described with reference to some specific embodiments thereof it should be understood that changes or modifications may be made by those skilled in the art without departing from the scope of the invention as defined in the appended claims.

Preferably murine oocyte is used, more preferably it is from the B6SJL strain. The two immunoglobulin chains of the anti-NGF antibody can be chimeric, obtained by assembling the variable murine regions of an anti-NGF monoclonal antibody, like Mab αD11, having human constant regions of K light and γ1 heavy chains, as described in the present invention or derived as such from the specific cDNA of the secreting hybridoma. The expression of the two antibody chains in the trangenic mouse is controlled by a strong viral promoter, as CMV-IE (Cytomegalovirus Immediate Early), which is expressed ubiquitously. Other ubiquitous promoters which can be used are RSV (Rous Sarcoma Virus) LTR, or SV40 (SV40-IE) early gene promoters. According to a further embodiment of the invention, in order to modulate the expression of the correctly assembled and functional antibody only in particular districts of the organism the two transgenes can be brought under the control of two different promoters. In order to express the functional antibody only in a particular district or control the same over the time, can be used however tissue-specific or inducible promoters which can be different for the two antibody chains.

The preparation of the transgenic animal carried out according to the method of the invention determines the expression of the functional antibody in the adult transgenic animal at levels about 2000 times higher than at the birth and this allows, on one hand, according to the method of the invention, to increase up to 80% the efficiency in the production of viable transgenic brood for both antibody chains, and on the other hand to express the phenotype resulting from the NGF activity only in the adult period, avoiding its neutralisation during the neuronal differentiation.

The transgenic mice obtained according to the method of the invention are able to express, at different level and in any case at amounts in the range from 50 to 500 ng/ml of serum, the functional chimeric antibody consisting of both the correctly assembled and secreted chains and said transgenic mice do not produce a lethal phenotype during the first post-natal

period, as opposed to the transgenic animal obtained by microinjection of the plasmids encoding for both the immunoglobulin chains in the same oocyte.

Time course experiments on the development of the neurodegenerative phenotype in the transgenic mice described in this invention reveal that, in the brain, the first signs of neurodegeneration are a cholinergic deficit and modification of some cytoskeletal proteins (2 months of age). The experiments of the authors of the invention pointed out that, in the periphery, the earliest signs of neurodegenerative pathology at the brain level are concomitant to an early (2 month age) tau hyperphosphorylation and amyloid deposition in the back or lower limb skeletal muscles. It is therefore within the scope of the present invention the use of the skeletal muscle monitoring for an early diagnosis of neurodegenerative diseases.

Further it is pointed out that the muscular system phenotype of the anti-NGF mouse can be reversed by NGF local administration. According to its further aspect, the invention therefore is directed to the use of NGF for the preparation of pharmaceutical compositions to be used for the therapy of muscular pathologies, as muscular dystrophy/atrophy. The administration of said neurotrophin can be carried out by different routes among which there are: intramuscular injection of NGF, for example recombinant NGF, dissolved in suitable physiological saline, or direct injection of plasmid or recombinant viral vectors, for example adenovirus, or by implant in the muscle of cells genetically engineered for the NGF secretion. The dose can depend on various variables as the specific activity of the protein, severity of the pathology to be treated, general conditions of the patient and in any case will be form 2 to 100 μg/kg of body weight.

Further it is found that the cholinergic deficit and the tau hyperphosphorylation in the cortex are reversed by direct infusion of NGF, or by infusion of agents that increase the production of NGF in the brain. Moreover, the neurodegenerative phenotype is reversed by intraventricular

infusion of a phage particle displaying a peptide recognized by the anti NGF aD11 antibody.

Description of figures

Figure 1. Production of anti-NGF transgenic mice. (A) DNA constructs for the production of the transgenic mice: light chain (upper panel) and heavy chain (lower panel) transgenes. CK and CH1-CH3, human constant region domains of light (K) and heavy (γ1) chains; VK and VH, light and heavy chain variable regions of the αD11 monoclonal antibody; CMV, cytomegalovirus promoter. (B) Crossing mice to generate mice expressing the functional anti-NGF antibody. VK-αD11 x VH- αD11 (VK: line of mice expressing the light chain of αD11 antibody; VH: line of mice expressing the heavy chain of αD11 antibody) (C) PCR analysis to detect the presence of VK (upper panel) and VH (lower panel) transgenes. The gels show 12 littermates born from homozygous VK (upper panel) or VH (lower panel) mice crossed to negative mice, to verify homozygosis of the single transgenic lines. As evident, all littermates carry the transgene. (D) Dot blot analysis of the four lines of mice expressing the heavy or the light chain. The upper panel was probed with a human heavy chain constant region probe and the lower panel with a human light chain constant region probe (see Methods). DNA samples in the upper panel: duplicates of VH-αD11 #D, wild type (WT, negative control) and VH-αD11 #C, single sample of human placental DNA (H.Pl.DNA, positive control). DNA samples in the lower panel: duplicate of VK-αD11#A, single samples of VK-αD11#B, WT (negative control), and Human placental DNA (positive control). (E) Levels of VH-αD11 (left panel) and VK-αD11 (right panel) mRNA in heart at P1 and P90 of mice from family #1, evaluated by phosphorimaging analysis, normalized to the β-actin mRNA (mean counts ± SEM).

Figure 2. Expression of functional antibodies in anti-NGF transgenic mice. (A) Expression of the recombinant VH in Purkinje cells of the cerebellum of VH-αD11 #C mice. Scale bar = 38 µm. (B) Expression of VH (left) and VK

(center) chains, in DRG (Dorsal root ganglia) of family #1 mice. The coexpression of the two chains in the same cells is shown in the right panel . Scale bar: 25 μm. (C) Level of recombinant αD11 in the serum (left) and in the brain (right) of family #1 and family #2 mice, at P1 and P90. The horizontal dotted line represents the detection threshold of the assay (0.1 ng/ml). (D) A transgenic control (transgenic for VH only) and a transgenic anti-NGF (family #3) mouse at P17. The transgenic mouse is much smaller than the control. (E) Body weight in anti-NGF mice (family #1 and family #2) (left) and transgenic control (right).

Figure 3. Phenotypic analysis of the central nervous system of anti-NGF transgenic mice A-H: Sections through the basal forebrain BF: ChAT-positive neurons in control (A) and anti-NGF transgenic mice (B). Cholinergic innervation to the frontal cortex, stained with anti-ChAT, in control (C) and anti-NGF transgenic mice (D). CP: caudate/putamen; FC: frontal cortex; GCC: genus corpus callosum. TrkA-positive neurons of the BF in control (E) and anti-NGF transgenic mice (F). p75-positive neurons in the BF of control (G) and anti-NGF transgenic mice (H). ChAT staining of hippocampal section in control (I) and anti-NGF transgenic mice (L). Timm's staining in hippocampal mossy fibers of control (M) and anti-NGF transgenic mice (N). Scale bar in (A-D; M-N)= 300 μm; (E-H) = 150 μm; (I-L) = 38 μm. The figures are representative of an analysis performed on 10 animals for each group.

Figure 4 Dilation of lateral cerebral ventricles Coronal sections stained with the cresyl violet method. Aged anti-NGF mic eshow dilation of laterla ventricle s(VL) (B) with respect to control mice (A). It has been osserved the atrophy of the septohippocampal nuclei (SHI) and of lateral septal nuclei (LS).

Figure 5 Atrophy of the cerebral cortex Coronal sections obtained at the level of the basal forebrain. The frontal cortex is atrophic in anti-NGF mice (B) with respect of to control mice (A).The white bar indicates the thickness

measured in the anatomical region. (C) Histogram comparing the status in transgenic (B) with respect to control (A) mice.

Figure 6 Atrophy of the hippocampus. In control mice hippocampi (Hp) are normal (A) with respect to transgenic mice (B). (C) Histogram comparing the status in transgenic (B) with respect to control (A) mice.

Figure 7 Neuronal apoptosis Tunel labeling of apoptotic cortical neurons. In control mice (A) there is no positivity, while in anti-NGF mice (B) there are many nuclei with DNA fragmentation.

Figure 8 Phosphorilation of tau protein Transgenic mice show a marked positivity for the N-terminal segment of tau protein (B), non phosphorilated tau (D) and hyperphosphorilated tau (F). In control mice there is no labeling (A,C,E). Labeling is localized in the cortex (E). Labeling for the N-terminal segment of protein tau in loclaized in some cells of the hippocampus (arrows).

Figure 9 Deposition of ß-amyloid protein plaques in the brain The antibody MN10 and the antibody against the amyloid precursor protein show plaques in control mice (A) and bigger plaques in transgenic mice (B). In both figure arrows point to plaques.

Figure 10 Western blot of brain extracts from anti-NGF and control mice Western analysis of brain extracts from anti-NGF and control mice. Blots were probed with mAbs YOL1 (anti-tubulin, as provided by Dr. Cesar Milstein) (A), AT8 (anti-phosphorilated tau) (B) and anti-APP (C). Blots are representative of 3 different experiment in which at least 3 control and 3 anti-NGF mice for each age were used. In each panel upper and lower rows represent blots from extracts derived from control and anti-NGF mice, respectively.

Figure 11 Accumulation of tau protein in the brain of anti-NGF transgenic mice. Insoluble tau protein accumulates in the brain of 15 months-old mice. Protein amount was checked using anti-tubulin antibodies (A). Tissues were sequentially extracted with RAB Hi-salt (B), RIPA buffer (C) and 70% FA (D).

RAB-insoluble tau, represented by the RIPA and FA fractions, accumulates in the brain of anti-NGF mice but not in control mice. B, C, and D were visualized with anti-tau antibodies 7.51. Lanes 1-2 refer to controls; lanes 3-5 to anti-NGF mice.

Figure 12 Amyloid deposits in the cortex of aged anti-NGF transgenic mice. Anti-APP immunoreactivity in cortical sections from anti-NGF (a) and control mice (b). The numerous extracellular amyloid deposits found in the cortex of anti-NGF transgenic mice show, at high magnification (c), a fibrillary nature. Scale bar in a, b = 75 μm; c = 25 μm.

Figure 13 Presence of compact amyloid plaques Compact amyloid plaques are observed in the subcortical white matter (A) and cerebral cortex (B) of 6 months-old anti-NGF mice. In 15 months-old anti-NGF mice plaques assume a fibrillary appearance with irregular contours (C) similar to that observed for typical plaques in AD human brains (D). Scale bars in A 150 μm; in B-D= 75 μm.

Figure 14. Immunostaining with the anti-tangle antibody mAb NFT200. a, b, c Sections through the parietal cortex (a, b) and the entorhinal cortex (c) of anti-NGF transgenic mice. The NFT200 antibody reveals the presence of tangles in pyramidal cells (arrows) and of dystrophic neurites (arrowheads). d, No labelling is seen in sections from transgenic control mice. Scale bar = 25 μm.

Figure 15. Neuron labelling by anti-NTF200 anti-tangles antibodies Anti-tangles NFT200 antibodies label neurons both in aged anti-NGF mice (G) and in AD human cortex (H). Scale bars 50 μm.

Figure 16 Time progression of neuron labelling by anti-MAP2 antibodies MAP-2 abnormal distribution in anti-NGF mice. At 2 (A), 6 (B) and 15 (E) months of age anti-MAP-2 (Sigma, St. Louis, MO, USA) labels the full length of cortical dendrites in control mice. In anti-NGF mice, a reduction of the number of labeled-dendrites and a re-distribution of the staining is observed.

The decrease in staining starts at 2 months of age (B) and proceeds with aging (D, F: 6 and 15 months of age, respectively). Scale bar = 100 μm.

Figure 17 Silver staining (E,F) Silver staining show the presence of extracellular deposits (asterisks) associated to dystrophic neurites (arrows) and tangle-like neurons (arrowheads). Scale bars 50 μm.

Figure 18 ChAT staining Staining for choline acetyl-transferase (ChAT) in the basal forebrain of anti-NGF transgenic mice (c) and control mice (d) 15 month old. Scale bar 200 μm.

Figure 19 Tau protein labelling At 1 month of age, AT8 antibodies stain neurons in all cortical layers of the entorhinal (A) and parietal (B) cortices of anti-NGF mice. In both cortices, the labeling decreases at 1.5 months of age (C). No difference is seen with control mice. At 2 months of age, AT8-positive neurons are observed in the entorhinal cortex (D), while only a few cells are faintly labeled in the parietal cortex (F). In age-matched control mice there is no labeling in neuronal bodies (E). In 6 months-old mice most neurons of the entorhinal cortex (G) express AT8 in their cell body and many of them also in dendrites (arrows). At this age, the parietal cortex (H) shows labeling both in neuronal perikarya and dendrites. No labeling (D) was observed neuronal bodies or dendrites of age-matched control mice (I). Scale bar 100 μm.

Figure 20. Tau protein labelling AT270 (A,B) and AT8 (C,D) antibodies labels pyramidal cells in hippocampal CA1 region in 15 month old anti-NGF mice (AT270:A; AT8: C). In age-matched control mice AT270 (B) and AT8 (D) antibodies label only axons. Scale bar 100 μm.

Figure 21 Tau protein labelling AT180 (Innogenetics, Gand BE) staining in cerebral cortex of anti-NGF mice increases with age. (E) Cortex of 1 month-old anti-NGF mouse, (F) 6 months-old anti-NGF mouse and (G) 15 months-old anti-NGF mouse. (H) 15 month-old control mouse. Arrowheads point to noneuronal, immunopositive cells. Arrows indicate dystrophic neurites. Scale bar 75 μm.

Figure 22 Tau protein mAb AT270 labelling. AT270 (Innogenetics, Gand, BE) immunolabeling in cerebral cortex of anti-NGF mice at (A) 1 month, (B) 1.5 months and (C) 2 months of age. No difference was observed when compared to control mice. Starting from 6 months of age in anti-NGF mice a specific labeling shows up in neurons of Layer II/III (D). The number of these positive cells increases in 15 months-old anti-NGF mice (E). with respect to control mice (F). At both ages no labeling is observed in age-matched control mice (F). Scale bar 100 μm.

Figure 23 Muscular atrophy. Transversal sections of skeletal muscles from transgenic mice and anti-NGF mice. Staining: hematoxylin-eosin. M. rectus medialis from the hindlimb of control (A) and Anti-NGF mice (B). M. gastrocnemius from the hindlimb of control (C) and Anti-NGF mice (D). M. tibialis anterioris from the hindlimb of control (E) and Anti-NGF mice (F).

Figure 24 Amyloid deposition in the muscle. Congo red staining show the presence of amyloid deposition in anti-NGF (B) and control mice (A).

Figure 25 Amyloid deposition in the muscle The immunoreactivity for amiloyd precursor protein in muscles from aged (15 month old) control mice is completely absent (A). In anti-NGF mice (B) there is an increase of labeling, coresponding to the brown precipitate, in the sarcolemma and cytoplasm of myofibers.

Figure 26 Phosphorylation of the tau protein in the muscle The immunoreactivity for hyperphosphorylated tau in muscles from aged control mice is completely absent (A). In anti-NGF mice (B) there is an increase of labeling, coresponding to the brown precipitate, in the sarcolemma and cytoplasm of myofibers.

Figure 27 Macrophage infiltration in the muscle Macrophage infiltration in the muscle. Longitudinal (A) and transversal (B) sections of muscles from aged anti-NGF mice. Immune cells, such as macrophages, are shown between myofibers.

Figure 28 Position of the nuclei in the myofibres of the anti-NGF mice In some myofibers from aged anti-NGF mice nuclei are localized at the center of the cell (arrows) and not at the periphery (asterisks).

Figure 29 Behavioral analysis of anti-NGF transgenic mice. (A) Nociceptive test in anti-NGF transgenic and transgenic control mice. Values are the mean ± SEM, n =10 for each group of animals (B) Open field test. (C) Rotarod test. *, $P < 0.05$. (D) Spatial learning curves for anti-NGF transgenic (n = 10, filled circles) and control mice (n = 10, open circles) mice in a radial 8 arms maze (four arms baited). Vertical bars are the standard errors. The number of arms entries necessary to find all four food pellets is reported as a function of time. (E) Retention test, 31 days after the end of the learning test. (F) Transfer test, started the day after the retention test.

Figure 30 Object recognition test The test reveals impairment in discrimination tasks. *, $P< 0.03$.

Figure 31 Phage ELISA. The data show that the stronger binder is the phage engineered to carry the peptide 18.

Figure 32 Immunohistochemistry Data reveals that the number of basal forebrain ChAT-positive neurons in 2-months old AD11 mice is decreased (B) with respect to transgenic control (A). The intraventricular administration of peptide 18 restores the number of ChAT-positive neurons (C) while the injection of a NT-3 related peptide does not allow the rescue of the number of cholinergic neurons (D). Hyperphosphorylated tau is localized in the soma of neurons in the entorhinal cortex of anti-NGF mice (F) with respect to control mice (E). The treatment with peptide 18 decreases the expresion in the soma (G) while the administration of the non related peptide is not affecting tau expression (H). Scale bar 320 mm.

Figure 33 Analysis of free NGF levels in the brain (A), blood serum (B) and submandibular gland (C) from control mice, AD11 mice, AD11 mice treated with phage carrying the peptide 18 or the NT-3 related peptide. (D) Total

number of ChAT-positive neurons in control mice, AD11 mice, AD11 mice treated with phage carrying the peptide 18 or the NT-3 related peptide.

Figure 34 Immunohistochemistry reveals that the number of basal forebrain ChAT-positive neurons in 2-months old AD11 mice is decreased (Fig. B) with respect to transgenic control (A). The intraperitoneal administration of LT4 restores the number of ChAT-positive neurons (C) while the injection the vehicle does not allow the rescue of the number of cholinergic neurons (D). Hyperphosphorylated tau is localized in the soma of neurons in the entorhinal cortex of anti-NGF mice (F) with respect to control mice (E). The treatment with LT4 decreases the expresion in the soma (G) while the administration of vehicle is not affecting tau expression (H). Scale bar 320 mm.

Figure 35 Analysis of free NGF levels in the brain (A), blood serum (B) and submandibular gland (C) from control mice, AD11 mice, AD11 mice treated with LT4 or vehicle. (D) Total number of ChAT-positive neurons in control mice, AD11 mice, AD11 mice treated with LT4 or vehicle.

Figure 36 A) Transgenic mice producing anti-NGF antibody (αNGF) show impairment of synaptic transmission Long-Term Potentiation (LTP) in visual cortex, induced by High Frequency stimulation (HFS) of the white matter, as compared with wild type mice. B) Exogenous supply of acetylcholine (ACh), to αNGF mice, is able to rescue LTP, in a concentration dependent manner. C) Four weeks of systemic administration of thyroid hormone (L-T4) to αNGF mice is not able to rescue plasticity by itself, but increase sensitivity to ACh, that at 10 μM concentration is already effective in the rescue of LTP as compared with L-T4 untreated αNGF mice.

Example 1 Production of anti-NGF transgenic mice and molecular characterisation

Transgene preparation

The recombinant chimeric antibody was obtained by assembling the sequences of DNA corresponding to the murine variable regions of the Mab

αD11 anti-NGF monoclonal antibody (Ruberti et al., 1993) (Genebank, access numbers: L17077/NID g310168: heavy chain and L17078/g310169: light chain, respectively) with the DNA corresponding to the constant regions of the human light K and heavy γ1 chains. Transcription units, corresponding to the chimeric light and heavy chains (figure 1A), containing at 5'end the Cytomegalovirus promoter and at 3'end the polyadenylation site of the bovine growth hormone (bGH), were cloned into the expression vectors pcDNAI-NeoVKαD11HuCK and pcDNAI-NeoVHαD11HuCγ, respectively. Then they were extracted using KpnI-ApaI and KpnI-XbaI restriction enzymes, respectively, purified and injected separately or in combination in the pro-nucleus of mouse B6SJL strain egg cells fecundated according to standard methods (for example see Allen et al., 1987). Two transgenic parents for the light (A and B family, low and high producer, respectively), two for the heavy (C and D family, low and high producer, respectively) and three for both (double transgenic) antibody chains were obtained, respectively. The latter parents, which express the antibody at a level of about 50 ng/ml, are unable to reproduce and therefore are unsuitable for the continuation of the study.

The molecular analysis of the transgenic parent mice (A, B, C and D families) was carried out by PCR (Figure 1C) or Dot Blot (Figure 1D) on genomic DNA extracted from tail biopsies as described in Piccioli et al., 1995. mRNA was extracted according to Chomcynzki and Sacchi, 1987, at different times form the birth and was analysed by RNAse-protection.

Preparation of the anti-NGF mouse

In order to generate transgenic animals for the functional antibody, consisting of both chains, two transgenic parents for the light (parents A and B) or for the heavy (parents C and D) chains were bred in different combinations (Figure 1B). Only breeding of A with D and B with C parents, which result in families 1 and 2 of double transgenic heterozygotic mice,

respectively, are fertile and generate viable animals with an over 80 % efficiency.

Characterisation of the anti-NGF mouse

The levels of the functional antibody of either light or heavy chains of the transgenic animals were measured by ELISA assays (Monlar et al., 1998), using a biotin labelled human anti-IgG secondary antibody, after 1:10 dilution of serum or brain homogenates (Piccioli et al., 1995) with PBS-2 % powder milk.

The levels of the anti-NGF chimeric antibody for families 1 and 2, measured in the serum and in cerebral tissue of adult animals (90 day-old) are higher than 100 ng/ml and 100 ng/mg, respectively. The values for family 2 are about two times higher than those for family 1. Soon after birth (1 day) the antibody levels are lower than the detection limit of the assay (0,1 ng/ml in the serum and 0,1 ng/ml in the tissues) (Figure 2C).

mRNAs specific for the chimeric VH and VK chains are expressed in different tissues among which brain, kidney, heart, muscle, liver and testicles. mRNA levels of both chains in the adult (90 day old) are about six times higher than in the newborn animal (1 day) (Figure 1E).

Therefore the high expression (1-2000X) of the anti-NGF functional antibody observed in the heterozygotous animal (double transgenic) is only partially due to the increase of mRNA levels.

Organ sections of anti-NGF mice are fixed by intracardiac perfusion of 4% paraformaldehyde in PBS, collected on a slide, preincubated in 10% foetal serum and 5% BSA, then used to detect by immunohistochemistry the expression of different antigens: particularly the co-expression of the light and heavy chains of the anti-NGF antibody were made detectable by biotinylated anti-human light or heavy chain (Amhersham), detected by HRP or AP-conjugated avidin-biotin (Elite Standard kits, Vector). The localisation at cerebral level is showed in figure 2A, while in figure 2B is revealed by

immunohistochemistry, demonstrating that the two chains of the chimeric antibody are co-expressed frequently.

Example 2 NGF phenotype knockout in anti-NGF transgenic mouse

The characterisation of the anti-NGF mouse phenotype was carried out at different levels: macroscopic, histological and molecular. At macroscopic level, during the first 3-4 life weeks anti-NGF transgenic mice do not show remarkable abnormalities, except an about 25% decrease of body weight compared to corresponding control mice (Figure 2 D and E). Usually experiments were carried out on anti-NGF transgenic animal of numerosity group n=6 with anti-NGF antibody levels from 50 to 300 ng/ml; as controls transgenic mice were used only for the antibody heavy chain (VH) (parent C or D), therefore not expressing the functional antibody.

At histological and molecular level the following differences, compared to normal mice, were observed, district by district: 1) central and peripheral nervous system, 2) muscular system and 3) spleen.

1) Central and peripheral nervous system

In the basal forebrain a reduction up to 57% in the number of acetyltransferase-positive neurons (ChAT) and a reduction of the expression level were observed, while in the hippocampus a reduction up to 70% of neurons is observed. In addition cells appear morphologically smaller. As to the peripheral nervous system the upper cervical ganglia are up to 45% smaller than control; also in this case cells appear morphologically smaller (Figure 3 A-B). The morphological and histological aspect of mouse brain expressing the anti-NGF recombinant antibody was analysed in transgenic 15-18 month-old mice («aged» animals), in combination with the presence of phenotypic markers of neurodegenerative diseases, as following: «neuronal loss» and apoptosis, expression of choline-acetyltransferase (ChAT) (Figure 3 A, B), determined by immunohistochemistry with anti-ChAT anti-serum (Chemicon International, Temecula CA, USA), ratio of phosphorylated to non phosphorylated tau protein (measured by immunohistochemistry or western

blot with specific antisera), presence of β-amyloid protein and of amyloid precursor protein (APP), (determined by immunohistochemistry with specific antisera),. The numerosity of the groups used for the experiments, except where otherwise indicated, was n=6 transgenic anti-NGF with transgenic anti-NGF antibody levels from 50 to 300 ng/ml; as control were used mice transgenic only for the antibody heavy chain (VH) (parent C and D), therefore not expressing the functional antibody.

Results can be summarised in the following points:

(a) Dilation of cerebral ventricles (Figure 4). The severity of the ventricular dilatation is associated to a remarkable atrophy of the cerebral cortex (Figure 5) and hippocampus (Figure 6).

(b) Neurodegeneration and neuronal loss. It is possible to visualise apoptotic cells in more severely damaged mice at cerebral level as pointed out by the TUNEL method (Figure 7). Apoptotic phenomena indicate a progressive cell death.

(c) Decrease of choline-acetyltransferase (ChAT) synthesis, (Figure 3 A-B and I-L), particularly in the basal forebrain. Namely no neurons positive for this marker in the nucleus of the medial septum are observed in analysed animals. The expression, when compared to that observed in younger (2-3 month-old) mice, is decreased. A lower expression is also observed for the two NGF-receptors, TrkA (Figure 3 E-F) and p75, in the basal forebrain (Figure 3 G-H). Particularly the decrease for the TrkA-positive is more remarkable than for p75-positive cells.

(d) Increase of the phosphorylation of tau protein (Figure 8). Using antibodies specific for the N-fragment of the tau protein [Alz-50 (Wolozin et al., 1986)] (Figure 8A and 8B), for the unphosphorylated tau protein [anti-Tau 1 (Grundke-Ipbal et al., 1986)] (Figure 8C and 8D), or for the same epitope of the phosphorylated tau protein [mAB AT-8 (Greenberg and Davies, 1990)] (Figure 8E and 8F), a remarkable generalised increase of the tau synthesis, mainly of the phosphorylated component thereof, was

detected by immunochemical methods. Used antibodies label cortical neurons which present a modified morphology, evidencing the presence of «neuropil threads», «ghosts» and «tangles». The labelling of the phosphorylated form of tau protein evidenced a remarkable increase of this protein also in the microglial cells which are activated in neurodegenerative processes.

In a further experiment the progressive increase of the hyperphosphorylated tau content in the brain of the anti-NGF mice was evaluated by biochemical analysis (Western blot analysis). Results were normalised for the total protein content using an antibody against tubulin (Figure 10A). The western blot analysis carried out using the antibodies against hyperphosphorylated tau (PHF-1 and AT-8) demonstrated that an increase of the phosphorylated tau content is present in 2 month-old animals and the tau content reaches a plateau 6 months after birth (Figure 10B). The biochemical analysis of the amyloid precursor protein demonstrated that the content of this protein increases from 6 months after birth (Figure 10C). Furthermore 15 months after birth two bands, corresponding to 120 kDa and 25 kDa, respectively, are observed (Figure 10C).

The presence of insoluble aggregates of tau protein was evaluated in 15 month-old anti-NGF mice. Brains were extracted sequentially using buffers with different extraction activity. Experiments evidenced that in the anti-NGF mice most hyperphosphorylated tau protein is insoluble (Figure 11).

Therefore the modification at the protein level of the tau cytoskeleton precedes the modifications observable at the amyloid protein level. Further the experiments evidence the presence of insoluble tau, which can be part of that component forming PHFs (paired helical filaments) which constitute the intracellular tangles and extracellular deposits in the Alzheimer disease. The results show a modified processing of the amyloid protein too.

(e) Amyloid plaques in the brain of 15 month-old anti-NGF transgenic mice. In another experiment the presence of amyloid plaques was detected using antibodies against the β-amyloid protein (4G8, Sentek, Maryland Heights, MO) and against amyloid precursor protein [APP (Glenner and Wong, 1984) (Chemicon International, Temecula, CA, USA)] evidence the presence of several plaques in both the paracingular cortex and neostriatum (Figure 9A and 9B).

The experiments were carried out using both immunohistochemical and Western blot techniques (see above). The results showed that, 15 months after birth, amyloid plaques are present in both the cerebral cortex and hippocampus of anti-NGF mice (Figure 12). These plaques cover a significant part of the enthorinal cortex surface, the percentage values being 21% of the surface compared to 0,5% in control mice. In other regions of the cerebral cortex the percentage of the surface covered by amyloid plaques is 10% and 0,1% in the anti-NGF mice and control mice respectively. The values are 4% and 0,1% in the hippocampus of the anti-NGF and control mice, respectively.

The plaque distribution and their morphology in the anti-NGF mice are entirely comparable to those observed in sections of patients affected by Alzheimer's disease (Figure 13). From above data it can be concluded that the anti-NGF mice display a high extracellular deposition of amyloid as plaque aggregates similar, as for morphology and distribution, to those observed in human brain sections of patients affected by Alzheimer's disease.

(f) Presence of neurofibrillary tangles in neurons of anti-NGF mice. The presence of intracellular tangles in brain sections of anti-NGF mice was showed using mAB NFT200, an antibody recognizing neurofibrillary tangles in AD brains (Innogenetics, Gand, BE). mAB NFT200 labelled many neurons distributed throughout the brain of anti-NGF mice (Figure 14a-c), but not in control animals (Figure 14d). The antibody detected the presence of

intracellular inclusions in dystrophic neurites too. In Figure 15 it is possible to compare the distribution of tangles in anti-NGF mice and in brain sections of Alzheimer patients.

The mAB NFT200 antibody reveals, in the brain of anti-NGF mice, aggregates similar to those observed in sections of human brain. This feature, indispensable to confirm the diagnosis of the Alzheimer disease in humans, was never detected up to now in other animal models partially reproducing this pathology.

Neurofibrillary tangles were also detected by silver staining. For these experiments a silver staining technique (Bielschowsky metohod), previously used to detect extracellular neuritic plaques and tangles in brain sections of Alzheimer patients, was used. In anti-NGF-mice this technique allowed to detect the co-existence of dystrophic neurites and extracellular fibrous material in the form of plaques (Figure 17E,F). These aggregates are evident in 6 and 15 month old mice.

The silver staining is a histological technique which allowed, independently from immunohistochemical techniques, to detect the presence of plaques consisting of extracellular deposited material and dystrophic neurites. In addition this technique allowed to detect the co-existence of these two modifications. The attempts to detect these modifications in other animal models for the Alzheimer disease has failed so far.

(g) Modifications of the distribution of MAP-2 (protein associated to microtubules). The protein associated to the microtubules (MAP-2) is part of the multiplicity of the proteins forming the cytoskeleton of neurons. The modifications of said protein were detected using the anti-MAP-2 antibody (Sigma, St Louis MO). 1 and 1,5 months after birth the observed distributions of the MAP-2 protein in the cortex neurons of anti-NGF transgenic and control mice were similar. 2 months after birth in the control mice the MAP-2 labelling is distributed throughout the dendrites lenghtwise (Figure 16A). At this age in the anti-NGF mice a decrease in the number of labelled dendrites

and a labelling redistribution in the dendrite lengthwise are observed (Figure 16B). 6 and 15 months after birth the number of dendrites is still decreasing in the anti-NGF mice. In the dendrites of these animals a clear labelling re-distributrion is also observed, which is localised in the proximal zone of dendrites (Figure 16D,F). In the same age control mice, the MAP-2 labelling is still distributed throughout the dendrites lengthwise (Figure 16C,E).

From these results it can be deduced that the NGF deprivation determines a modification in the distribution of the cytoskeleton proteins of the cortical neurons. This modification could be part of the neurodegenerative phenomena leading to the occurrence of the Alzheimer disease.

(h) Time course of the neuropathology in anti-NGF mice. Experiments to evaluate the occurrence of modifications in the different phenotype markers were carried out. This time course is summarised below and in Table 3:

1. The decrease of cholin-acetyltransferase(ChAT)-positive neurons, previously described (Ruberti et al., 2000), continues 2 months after birth and reaches a plateau 6 months after birth whereupon a 90% reduction in the number of positive neurons in the medial septum (Figure 18C,D) is observed.

2. The determination of the somatodendritic distribution of the tau protein in hyperphosphorylated form was carried out by different antibodies and, in the anti-NGF mice, showed as follows:

- 2 months after the birth only the enthorinal cortex presents modification detected by mAB AT8 (Figure 19),
- these modifications extend to other regions of the cerebral cortex and hippocampus (Figure 19 and Figure 20) from 6 months after birth,
- these modifications are detected also by other antibodies, different from AT8, i.e. PHF-1, AT180 and AT270 mAbs.

3. The AT8 antibody, used together with extraction techniques, shows that most of the tau protein extracted from the anti-NGF mice brain is insoluble.

4. The cytoskeleton modifications concern not only the protein tau but also MAP-2 protein and start 2 months after birth.

5. The tangle-like inclusions are present only 15 months after birth, whereas the dystrophic neurites are detected already 6 months after birth.

6. The DNA fragmentation is observed only 15 months after birth. In conclusion the anti-NGF mice present a time course of the neurodegeneration starting from the cholinergic deficit and modification of some cytoskeleton proteins. The spatial progress of the pathology is similar to what observed in brain form Alzheimer patients.

Table 3

PHENOTYPIC MARKERS	BRAIN AREAS	Age (months)				
		1	1.5	2	6	15
ChAT reduction		-	-	+	++	++
Hyperphosphorylated tau in the somatodendritic compartment						
AT180	Entorhinal cortex	-	-	-	+	++
	Parietal cortex	-	-	-	+	++
	Occipital cortex	-	-	-	+	++
	Hippocampus	-	-	-	-	-
AT270	Entorhinal cortex	-	-	-	+	++
	Parietal cortex	-	-	-	+	++
	Occipital cortex	-	-	-	+	++
	Hippocampus	-	-	-	-	+
AT8	Entorhinal cortex	-	-	+	++	++
	Parietal cortex	-	-	-	+	++
	Occipital cortex	-	-	-	+	++
	Hippocampus	-	-	-	+	++
Insoluble Tau		ND	ND	ND	ND	++
Abnormal subcellular localization of MAP-2		-	-	+	++	++

Neurofibrillary tangles		-	-	-	-	++
Amyloid plaques		-	-	-	+	++
Inclusions as revealed by silver impregnation		-	-	-	+	++
Dystrophic neurites*	Entorhinal cortex	-	-	-	++	++
	Parietal cortex	-	-	-	+	++
	Occipital cortex	-	-	-	+	++
	Hippocampus	-	-	-	-	-
DNA Fragmentation	Cerebral cortex	-	-	-	-	+
	Basal forebrain	-	-	-	-	-

+ : qualitative measure of each phenotypic marker; ND: not determined; * : as detected by silver and immunohistochemical impregnation by hyperphosphorylated anti-tau antibodies and «tangles».

The analysis showed that the neurodegenerative pathology at the brain level is preceded by an early (2 months after birth) tau hyperphosphorylation, as detected by the AT8 antibody (which is able to bind to a phosphorylated tau epitope, selectively expressed in Alzheimer affected patients) and amyloid deposition in the back or lower limb skeletal muscles (see in the following). It is to be pointed out that the association of Alzheimer disease with inclusion body myositis in humans is already known.

In summary transgenic mice expressing the anti-NGF antibody resemble at the level of the Central and Peripheral Nervous System many pathological modifications typical in neurodegenerative diseases, particularly Alzheimer disease.

2) Muscular system

Mice evaluated (n=15) from 45 to 60 days after birth, at a macroscopic level stagger, due to an abnormal position of the rear legs and support of toe tips and often present backbone scoliosis. The anatomical analysis shows a size reduction of the back longitudinal skeletal muscles, flexor and adductor

of the rear limbs, feature not observable in other muscles, for example in the corresponding muscles of the front limbs. Some deficits were better characterised and detailed as follows:

a) muscular dystrophy, characterised from the morphological and histological point of view. The atrophy of the muscular fibres is present, in all the considered animals (n=15), for the muscles which allow the movements of the backbone and aid the stability of the connections of each other vertebra (longest muscle for the backbone and inter-vertebral muscles, respectively). Further in all the animals the reduction of the diameter of the muscular fibres (up to 50%) is observed in 70% of the fibres: in the adductor (leg medial rectus, large and small adductor), leg flexor (outer, medial and inner ischiotibial) and metatarsus extensor (gastrocnemius and soleus muscles). On the contrary the atrophy is not present at the level of the metatarsus flexor muscles (front tibial and phalanx extensor muscles) and it is less evident in the front limb extensor muscles (brachial triceps muscle). All these differences are showed in Figure 23. Further every dystrophic muscular fibre show also a remarkable vacuolization (Figure 23B-E) and a more intense staining by haematoxylin/eosine.

b) scoliosis, in some animals (n<6), in some cases associated with an incomplete development of the vertebral bodies.

c) muscular atrophy, typified at molecular level as follows:

c.1) re-expression of the low affinity NGF receptor (p75). It is particularly clear in some muscular cells also exhibiting modifications in the distribution of nicotinic receptors at the level of the neuromuscular junctions.

c.2) decrease in the number of nervous peptidergic endings at the level of neuromuscular junctions. This decrease was detected by antibodies against the calcitonin gene-related peptide [CGRP (Gibson et al., 1984)].

c.3) absence of the aggregation of the acetylcholine receptors in the plasmatic membrane of the muscular cells, as detected by the irreversible binding of alfa-bungarotoxin (Changeux, 1991), caused by the reduced

innervation of the muscular fibre. The distribution of the muscular cells exhibiting such a modification gives the muscles of the transgenic mice a characteristic mosaic pattern.

c.4) increase of the immunoreactivity for dystrophin, detectable, in the above described cells exhibiting molecular modifications, by immunohistochemistry using D-8043 antibody (Sigma). Dystrophin is a protein of the skeletal muscular cell involved in muscular contraction and in the aggregation of the cholinergic receptors. It is already known that an increase in the dystrophin synthesis occurs concurrently with muscle denervation.

c.5) ATPase decreased metabolism due to the lack of the nerve trophic effect.

c.6) remarkable deposition of amyloid substance, detected by a characteristic ring cytoplasmic staining by Congo Red (Figure 24A-B). The presence of amyloid and particularly β-amyloid was detected also by immunohistochemistry against the β-amyloid precursor protein (Figure 25A-B) in «aged» mice.

c.7) phosphorylated tau protein in the muscles of the aged mice (age from 15 to 18 months). Figure 26A-B.

c.8) presence of various muscular fibres with nuclei located at the center of the fiber, rather than below the sarcolemma in aged anti-NGF mice (Figure 28). Histological assays detects an infiltration of immune type cells, probably macrophages, among the muscular fibres (Figure 27A-B).

The presence of deposits of the β-amyloid and hyperphosphorylated tau protein and in addition nuclei located in the middle region and macrophages infiltration, is related to what observed in inclusion body myositis (IBM), a pathology strictly correlated with the Alzheimer disease.

3) Spleen

At the anatomical level the localisation of the sympathetic innervation is distributed in the germinal centre and marginal zone, rather than in the

proximity of the central artery, as in the control mice. The recovery of the viable splenocytes is reduced by one order of magnitude (2-3 x 10^6 vs. 2-3 x 10^7 of the controls) in the anti-NGF transgenic mice, as observed by flow cytometry. Functionally a reduction of the number of the IgG positive lymphocytes can be observed and a fair increase of the IgD positive lymphocytes, as measured after incubation (30', 4°C) of the splenocytes with FITC anti-IgG (Sigma), IgM, IgA, IgD mouse (Pharmingen) labelled primary antibodies and analysis by Coulter Epics Elite Esp Flow Cytometer at 488 nm. Furthermore in the red pulp DNA fragmentation, indicating apoptosis, can be detected, consistently with the reduced recovery of viable splenocytes.

Example 3 Analysis of the behaviour of anti-NGF transgenic mice

The analysis was carried out on 12-18 month old animals (n=6), selecting animals without evident gait anomalies. The following anomalies, resumed in Figure 29, with respect to the control animals were detected:

- Increase of the latency time for the heat sensitivity, changed from 3» for the control mice to 16» for the anti-NGF mice, as measured according to the hot plate nociceptive assay, already described in Eddy et al., 1953.

- Spatial orientation. This was measured by the radial labyrinth test, carried out as follows: the animals were located in 8 arm radial labyrinth and free to feed themselves for 5' and familiarise with the labyrinth over two days. For the test the same four arms were filled with food every day; at the beginning of each test the mice were left at the centre of the labyrinth, free to explore it: the test was terminate if the food was finished or 25 entries were observed into the arms of the labyrinth: the tests were repeated twice a day over 14 days, made mistakes (short and long term memory mistakes) and taken times being measured. The starting and final learning levels were evaluated using the average of the mistakes made over the first and last three days. The anti-NGF mice exhibit a higher number of errors during the working memory learning over the first three days, in fact the learning plots

are significantly different (two way RMANOVA test, $p<0,05$), however the final learning level is not different from that of the control mice.

- Ability in maintaining the acquired notions. The anti-NGF mice do not maintain the acquired notions at 31st day after the final learning step, as measured by the same radial labyrinth test. The learning plots were compared with the two way ANOVA test (treatment x time) and the significance of the differences evaluated by T-test.

- Deficit in the ability of learning transfer into other situation, as measured by the radial labyrinth test, using food filled arms, different from those used in the learning step. The anti-NGF mice exhibit a clear learning deficit ($p < 0,01$ in two way RMANOVA test) in comparison to the control mice, also after 5 learning days. The differences resulted mainly from a higher number of short term memory errors (T-test, $p<0,006$).

- Short term memory test (object discrimination test). According to this test the mice explored 2 white cubes over 10 minutes. Then one cube was coated with white and black chess painted paper. One hour after the end of the first trial the mice were allowed to come again in contact with the cubes and explore them over additional 10 minutes. The anti-NGF transgenic mice were not able to distinguish between the two cubes coated with differently coloured papers (Figure 30). Therefore the anti-NGF mice show a decrease in the short term memory, not being able to memorise and distinguish between the two differently coloured cubes.

Example 4 Reversibility of the muscular dystrophy in anti-NGF mice by NGF local administration

All the experiments were carried out on 45 day old mice, when the serum level of the anti-NGF antibody is still not at the highest level (observed 60 days after the birth). The NGF was administered locally by different methods: (a) by intramuscular injection of NGF, (b) by intramuscular injection of a viral recombinant vector (adenovirus) encoding for NGF cDNA or (c) by the implant of NGF secreting fibroblasts. All the administration

routes included the injection or implant in the gastrocnemius muscle, one of the skeletal muscles affected by muscular dystrophy. The injections and implants were carried out on the right leg, while the gastrocnemius muscle of the left leg was used as control. A) NGF was injected as pellet, consisting of diazocellulose mixed NGF in borate buffer at pH 8,0 for 72 hours and following neutralisation by glycine saturated solution (Hendry, 1982). This method allows the exact localisation and slow release of this neurotrophin. Different NGF concentrations were used, comprised in the range from 100 μm and 2 mg for each animal. For the administration of cDNA according the method b), 10 μl of the adenoviral vector solution corresponding to 10^7 pfu/ml were injected in the gastrocnemius muscle. In the control animals a recombinant adenovirus containing Escherichia coli Lac Z reporter gene was injected. Both in this and in fibroblast injection experiment (see later) NGF production was constant at least over one month, allowing the reversibility of the phenotype to be observed.

According to method c) fibroblasts genetically modified to secrete NGF by infection with a retroviral vector encoding for cDNA of this neurotrophin, according to the method described by Gage et al. (1990), were implanted in the muscle. This allowed to obtain an in situ NGF production equal to 100 ng/10^6 cells/day. The fibroblasts were injected after re-suspension in sterile physiological saline at a 2 x 10^5 cells/μl concentration. The injection volume was 10 μl/animal.

To verify the effect of the NGF administration the animals were sacrificed 7, 15 and 30 days after the injection. The injected and contralateral muscles were collected and analysed by histological and immunohistochemical techniques to verify the attenuation of the dystrophy and the restoration of usual innervation. Thus it was verified that in all the injected animals the values of the muscular fibre diameter were again similar to those of the control animals. Furthermore their morphology and cholinergic and peptidergic innervation re-assumed an usual appearance.

Example 5 Restoration of the CNS phenotype in anti-NGF mice by NGF local administration

The restoration of the cholinergic phenotype in the basal forebrain was achieved by two different approaches. In a first set of experiments, NGF was delivered using slow releasing minipumps (Alzet, USA) A rubber capillary tube was inserted in the lateral ventricle and then connected by means of an osmotic minipump to a «pocket» of the subcutaneous layer. This pump was filled by NGF (30–100 μg) diluted with Ringer-Locke physiological saline. Experiments were performed in 2 months-old transgenic mice and controls.

In another set of experiments, animals of the same age of previous ones were treated by an implant of fibroblasts genetically modified to secrete NGF. The fibroblasts were injected in the lateral ventricle at a 2×10^5 cells/μl concentration. The injection volume was 1,5 μl/animal. This second method allowed to verify, by ELISA, the constancy of NGF production over 4 weeks after the implantation. The analysis of the cholineacetyl transferase expression (ChAT) in the nuclei of the basal forebrain and the analysis of the behaviour of these mice allowed the phenotype reversibility to be observed in the anti-NGF mice at the level of the cholinergic system.

As a whole these results confirm that the NGF administration is able to correct the muscular and cholinergic deficit observed in the anti-NGF mice.

Example 6 Rescue of the cholinergic phenotype in anti –NGF mice

Since at 2 months of age cholinergic deficit is one of the first signs of neurodegeneration, the authors evaluated the possibility of restoring the cholinergic neuronal loss using two different approaches. The first one is based on the systematic injection of L-thyroxine (LT4), an hormone that is known to produce an increase of NGF synthesis and a consequent increase of ChAT in neurons of the basal forebrain (Luesse et al., 1998). In a second series of experiment a phage library was used to identify a peptide that has

a sequence different from NGF and could compete for the binding site of the monoclonal antibody αD11. The phage carrying the selected peptide was intraventricularly injected in anti-NGF mice brain. The aim of these treatments was to increase the availability of free NGF to target cells. The effects of LT4 and the synthetic peptide were analyzed both at the level of cholinergic neurons of the basal forebrain and of NGF synthesis in brain, submandibular glands and blood serum.

MATERIALS AND METHODS

Animals. Control (VH only) mice and anti-NGF transgenic mice were generated following the injection of DNA fragments containing the transcriptional units of the light and heavy chain of chimeric αD11 antibody, placed under transcriptional control of the ubiquitous human cytomegalovirus early region promoter (Ruberti et al., 2000). Mouse genotype was verified by PCR analysis of tail DNA. Animals were maintained on a 12-h light, 12-h dark cycle and fed ad libitum. All experiments were performed following European Community rules for animal care.

Phage-display peptide technology. To display large collections of peptides on the surface of phage, mixtures of oligonucleotides containing regions of randomized sequence have been inserted into the the N-terminus of the product gene encoding pVIII protein. A seven amino acid random peptide library exposed on the major coat protein VIII with a diversity of 2.2 107, kindly provided by G. Cesareni , was used in these experiments.

Biopanning. Polystyrene beads were incubated overnight at 4°C with 1µg/ml of αD11 in 0.5 M carbonate buffer pH 9.6. After washing with PBS and H_2O_2, the beads were blocked by incubating with TBST (50 mM Tris/HCl pH 7.5, 150 mM NaCl, 0.5% Tween 20) and 10 mg/ml bovine serum albumine (BSA) for 4 hrs at 4°C and washed briefly in TBST.

One bead was then added to 1 ml of TBST containing 1 mg/ml BSA, 2.5 10^{10} pfu/ml of UV-killed (defective in replication) phage particles (M13) and incubated with rotation at 4°C for at least 4 hrs. Then, the pVIII phage

library was added (210^9 pfu/ml) and incubated overnight at 4°C. Supernatants were collected and stored. After washing in TBST the bead was transferred into a glass tube containing 1 ml elution buffer (0.1N HCl pH2.2, 1 mg/ml BSA) and incubated under strong agitation at 37 °C for 10min. The eluted phages were transferred to a polypropylene tube and neutralized by adding 100 □l of 2 M Tris/HCl pH 9.00. As a final step, supernatants and adsorbed phages were titred. A maximum of 3 rounds of biopanning was performed.

Phage amplification. The eluted phage particles were plated onto a bacterial layer overnight at 37°C. The next day, phage plaques were scraped by adding 5 ml of (LB). The obtained suspension was shaked at 37 °C for 30 min, spun at 4,000 r.p.m. for 15 min. The phage particles were precipitated by adding 1/5 volume of PEG NaCl (20% polyethylene glycol 6000, 2.5 M NaCl) to the supernatant, mixed and left stand for 1 h at 4°C. After spinning at 11,000 rpm for 30 min, the pellet was resuspended in 1 ml of water, transferred into a 1.5 ml eppendorf tube, kept for 10 min at 70°C, spun for 5 min in microcentrifuge. The supernatant was transferred to a new tube, PEG/NaCl was added, mixed and left stand for 20 min at 4°C. After spinning, the supernatant was discarded and the pellet was resuspended in 1.5 ml of water, centrifuged for 2 min, filtered through a 0.45 μm sterile filter and titred.

Plaque immuno-screening. Adsorbed phage particles were plated on LB and incubated for 3 h at 37°C, then a nitrocellulose filter was overlaid on the plate and left overnight at 37°C and then for 1 h at 4°C. Filters were washed 4 times in 5% non-fat dry milk/PBS for 30 min at room temperature under agitation. The filters were then incubated with αD11 (1 μg/ml in 5% milk/PBS) overnight at 4°C, washed in 0.1% Tween/PBS at 4°C. The reaction was revealed using an alkaline phosphatase conjugated anti-mouse antibody (Sigma), for 4 hours at 4°C, followed by incubation in NBT and BCIP.

Rapid Sequencing of ssDNA phages. The plaques that showed a strong and positive reaction on nitrocellulose filter were sequenced. SsDNA templates were prepared as described[8] and resuspended in 10 µl of H_2O_2. R156 (5' AACCATATATTCGGTCGCTGAGGC3') has been used as primer oligonucleotide for phage sequencing.

Single ssDNA template were subdivided in 4 wells of a 96-well plate, incubated with 2 µl of annealing mixture (6 µl H_2O_2, 2 µl sequenase reaction buffer 5X, and 1 pM primer oligonucleotide) at 55°C for 15 min. Then, 2 µl of reaction mixture (7 µl H_2O_2, 0.5 µl ^{35}S-ATP, 0.4 µl 0.1M DTT, 0.4 µl labelling mix, 0.25 µl USB Sequenase 5.0) were added to each well. Sequencing was performed using sequenase USB kit, according to the manufacturer instructions.

Phage ELISA assay. Ninety-six well plates were coated with 100 µl of rat anti-pIII monoclonal antibody (57D1, gift by P. Delmastro) diluted in coating buffer (1µg/ml 50 mM carbonate buffer pH 9.6) and incubated overnight at 4°C. Palts were washed in PBST and blocked in 5% milk/PBST for 1h at 37°C on a rocking platform. After 1 brief wash, a mixture of cleared phage supernatant (50 µl) and 50 µl of 5% milk/PBST was added and incubated for 1h at 37°C. The plates were washed and 100 µl of αD11 antibody dilution (1µg/ml in 5% milk/PBS) were added and left overnight at 4°C. After washing, an alkaline phosphatase conjugated anti-mouse antibody was addedd and left at 4°C for 4 hrs. The reaction was developed by adding 200 µl of the substrate solution (10% dietanolamine, 5 mM $MgCl_2$ pH 9.6) for 2 min followed by an incubation at 37°C for 1 h in 100 µl of developing solution (1mg/ml NTB in 10% dietanolamine, 5 mM $MgCl_2$, pH 9.6).

Peptide 18 treatment. Prior to the injections, phages carrying the peptide 18 or a peptide raised against NT-3 (negative control) were amplified by infecting 2.5 ml of an overnight colture of DH5alpha F' bacteria with 5 ml of phage supernatant in 1 liter of 2TY. After an overnight incubation

at 30°C, bacteria were removed by centrifugation at 6000 rpm for 20 min at 4°C. Phage particles were precipitated by adding 1 volume of PEG/NaCl (20% PEG6000, 2.5 M NaCl) to 5 volumes of the supernatant. After mixing, the solution was left stand overnight at 4°C and spinned at 6000rpm for 30 min. The pellet was resuspended in 40 ml of H_2O_2, kept at 65°C and then spun for 5 min at 11000 rpm.

One volume of PEG/NaCl was mixed to 5 volumes of the phage containing supernatant and incubated for 2 hrs at 4°C. Afetr spinning at 11000 rpm for 5 min., the pellet was resuspended in PBS, spun again to remove cell debris and filtered through a 0.22 μm sterile filter. The phage suspension was then titred by infecting 100 μl of an overnight colture of DH5α F', mixed with LB top agar and plated on LB plates. Phages carrying the carrying the peptide 18 or a peptide binder of anti-NT-3 (10^9 –10^{12} pfu/μl) were injected in both lateral ventricles. Phages were injected at postnatal day 53 (P53) and mice were killed 1 week after the injection. For the injection, mice were anaesthetised with 2,2,2-Tribromoethanol (0.2 ml/10 g body weight of a 1.2% solution). One μl of phage suspension was injected using a 17 gauge needle connected to a 10 μl Hamilton by a polyethylene cannula, 1 mm lateral and 1 mm anterior to Bregma. After the injection, animals were treated with ampicillin (1mg/kg) every day, in order to prevent any bacterial infection. Experiment were performed three times for a total number of animals equal to 14 for each group of treatment.

Phage detection. Levels of phage were analyzed in the brain of each animal using brain slices containing the basal forebrain. Samples were sonicated twice for 10 sec. in 200 μl of PBS. Fifty μl were used for direct titration. One hundred and fifty μl were used to infect a colture of DH5α F' to amplify phage particles and after an overnight incubation at 30°C, phages were precipitated and titred.

L-thyroxine treatment. L-T4 was administered according to schedule and dosages which were shown previously to produce the maximal effects,

at least within submandibular gland of normal mice (Raynaud, 1964). L-T4 was administered intraperitoneally (10 μg in 0.1 ml of 0.1 mM sodium carbonate in phosphate buffered saline (PBS)) daily from P45 to P62 (n=16). Anti-NGF mice injected with vehicle (n=15) were killed at the same time as the experimental animals.

Immunohistochemistry. Transgenic controls and anti-NGF mice were anaesthetized with 10.5% chloral hydrate/saline (8 μl/g body weight), and transcardially perfused with ice-cold PBS. Brains were removed, fixed in 4% paraformaldehyde/PBS for two days at 4 °C and cryoprotected in 30% sucrose overnight. Coronal sections (40 μm thick) were collected in 1% paraformaldehyde/PBS, preincubated in 10% fetal calf serum and processed for detection of different antigens using avidin-biotin horseradish peroxidase Elite Standard kits (Vector laboratories, Burlingame, CA). The following primary antibodies were used: anti-choline acetyl transferase (ChAT; Chemicon, Temecula, CA, 1:500) and the monoclonal antibody against phosphorylated tau (AT8; Innogenetics, Gand, Belgium). Parallel sections from transgenic and age-matched transgenic control (VH only) mice were processed at the same time.

Quantitative stereology. The volume of basal forebrain and the number of BFCNs was evaluated in 9 anti-NGF transgenic mice and 8 transgenic controls by using a stereological approach (Ruberti, 2000).

Determination of free NGF. The levels of free NGF (i.e. NGF not bound to the transgenic antibodies) in the different tissues was determined by an ELISA assay. This assay exploits the property of αD11 antibody to recognize NGF in a two-site ELISA format16. Samples of blood serum or of tissue extracts (derived as in Molnar et al., 1998) were added to wells coated with mAb αD11 (coating concentration of 5 mg/ml). After incubation for 2 hours at r.t. and extensive washing with PBS-0.05%Tween 20, followed by PBS, free NGF, not engaged with transgenic αD11, was detected using an affinity-purified rabbit anti-NGF polyclonal antiserum.

RESULTS

Screening of phage display libraries. The data obtained by the successive rounds of biopanning showed that a positive selection was occuring (data not shown). After plating and the formation of individual plaques, the immunoscreening procedure allowed to select only those peptides that bind the antibody in the same region as NGF. The □D11 antibody showed 70% of positive clones in filter immunoassay.

Positive phage clones were analyzed by the single strand DNA sequencing. The positive phage clones were sequenced and re-tested using dot blot experiments towards the αD11 antibody. At least twenty were analyzed . A group of phages selected against αD11 was tested by ELISA to identify the phage clone with a stronger binding activity (Fig. 31) that resulted to peptide 18, having the following sequence RGSRHDL. An immunoscreening in which the binding of the antibody to phage was competed by NGF was performed and demonstrated that selected peptides could compete with NGF for the binding to the antibody.

A parallel selection was performed with anti-NT-3 antibodies to exclude the binding to non relevant antibodies.

Peptide 18 intraventricular injections. The analysis of the number of ChAT positive neurons in the basal forebrain of anti-NGF mice revealed a decrease around 40% with respect to transgenic control mice (Fig. 32A,B, Fig. 33D). The intraventricular administration of the phage carrying the peptide 18 restored the number of ChAT positive neurons in the basal forebrain of anti-NGF mice to normal values (Fig. 32C, Fig. 33D) while the injection of the peptide binder of anti-NT-3 did not restored the normal values of ChAT-positive neurons (Fig. 32D, Fig. 33D).

In anti-NGF mice hyperphosphorylated tau is localized in the soma of neurons of the entorhinal cortex (Fig. 32F) with respect to control (Fig. 32E). The rescue of the cholinergic phenotype is not paralleled by the disappearence of AT8 labeling in the somatic compartment of neurons of the

entorhinal cortex both in animals treated with peptide 18 (Fig. 32G) and in those treated with the non related peptide for anti-NT-3 (Fig. 32H).

Concerning the values of free NGF, they increase only at the level basal forebrain, corresponding to the brain area next to the injection site. (Fig. 33A). In blood serum (Fig. 33B) and submandibular gland (Fig. 33C) NGF levels were equal to animals treated with the non related peptide binder of anti-NT-3.

LT4 treatment. The intraperitoneal injection of LT4 produces an increase of the number of ChAT-positive neurons in the basal forebrain of anti-NGF anti-NGF mice (Fig. 34C, Fig. 35D). The number of cells was equal to that observed in transgenic control animals (Fig. 34A, Fig. 35D) and was 40% higher than that observed in not-treated anti-NGF mice (Fig. 34B, Fig. 35D) or in animals treated with vehicle (Fig. 34D, Fig. 35D).

In anti-NGF mice hyperphosphorylated tau is localized in the soma of neurons of the entorhinal cortex (Fig. 34E,F). The rescue of the cholinergic phenotype was paralleled by the disappearence of AT8 labeling in the somatic compartment of neurons of the entorhinal cortex (Fig. 34G), while the administration of the vehicle did not affect the expression of tau (Fig. 34H).

The analysis of NGF levels in brain, blood serum and submandibular gland revealed that, in anti-NGF mice treated with LT4, the amount of free NGF was higher than that detected in animals treated with vehicle (Fig. 35A, B, C). The values obtained reached almost the levels detectable in transgenic control animals.

Example 7 Electrophysiological data

Dysfunction of basal forebrain cholinergic neurons affects experience-dependent plasticity (Gu & Singer, 1993) and is involved in major human cognitive impairments (Dunnett et al., 1991). Recent in vivo experiments indicate that NGF modulates cortical plasticity in the sensory cortex both during postnatal development (Domenici et al., 1991; Domenici et al., 1993;

Domenici et al., 1994) and in adulthood (Gu et al., 1994). Experiments on rat visual cortex slices showed that NGF controls the expression of long term potentiation (LTP), an action mediated by the cholinergic system (Pesavento et al., 2000).

The authors decided (i) to analyze, at the neurophysiological level, the effects of chronic NGF deprivation on BFCNs and cortical experience-dependent plasticity; and (ii) to attempt the rescue of the cholinergic deficit and hence the functional alterations in the cerebral cortex, by the administration of NGF and /or cholinergic agonists.

MATERIALS AND METHODS

Visual cortex slices preparation and electrophysiological recordings. Postsynaptic field potentials were recorded in rat in vitro slices containing visual cortex, following previously described methods (Kirkwood and Bear, 1994). Anti-NGF and transgenic control mice were deeply anesthetized with urethane and then decapitated. The brain was rapidly removed and visual cortex slices (400 μm) prepared and superfused in a submerged recording chamber at 33 °C with gassed artificial cerebrospinal fluid (aCSF) at a rate of 4 ml/min.

Extracellular field potentials in the inferior half of cortical layers 2/3 were recorded with an electrode filled with a 2M NaCl solution and evoked by stimulation of the white matter containing geniculo-cortical fibers, using a bipolar concentric stimulating electrode. The amplitude of the maximum negative field potential in layer 2/3 was used as a measure of the evoked population excitatory current. LTP was induced by 3 trains of high frequency stimulation (HFS, 100Hz, 1s). Experiments were performed at different postnatal ages 6 months, when the functional maturation of the visual cortex is completed. Acetylcholine dependence of LTP was investigated by locally delivering through the recording pipette. The amplitude of the maximum negative field potential in layer 2/3 was used as a measure of the evoked population excitatory current. The magnitude of both LTP was measured

starting 30 min after the end of the corresponding conditioning protocol. Data for each experimental group were pooled and expressed as percentage change from control baseline, PCCB ± S.E.M. Statistical comparison was done by applying a t-test and Mann-Whitney Rank Sum Test between baseline and LTP mean values.

RESULTS

In adult anti-NGF mice, chronic deprivation of NGF determined a decrease in cortical experience-dependent plasticity. In anti-NGF mice, slices containing the visual cortex do not display a particular form of synaptic plasticity, Long Term Potentiation (LTP) (Fig. 36A).

The abnormality in LTP was rescued by adding in the recording chamber acetylcholine (100□□M). At lower concentration (10 □M), acetylcholine was ineffective (Fig. 36B).

L-thyroxine is an hormone that increases the levels of endogenous NGF in mouse brain (Giordano et al., 1992). The treatment of anti-NGF mice with L-thyroxine (12 □g/animal/day) for 4 weeks allowed only a mild rescue of synaptic plasticity (Fig. 36C). However, in these animals a lower dose of acetylcholine (10 □m), that was per se ineffective, is sufficient to obtain normal levels of LTP (Fig. 36C).

It can be concluded that:

1) Chronic NGF deprivation determines a decreased synaptic plasticity in the visual cortex of anti-NGF mice.

2) This deficit can be rescued by acetylcholine, that can be linked to the decreased cholinergic innervation of the cortex (Ruberti et al., 2000).

3) Treating mice with L-thyroxine restores NGF levels in the cortex. Although NGF is not sufficient to restore synaptic plasticity in the cortex, it facilitates acetylcholine actions, allowing decreasing acetylcholine amount to be delivered.

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CLAIMS

1. A process for the production of tungsten-copper composite powders suitable to be pressed and sintered and having a copper content from 5 to 35% by weight, said process comprising the following steps:

a) suspending an elemental tungsten powder in a liquid polyol or in a mixture of liquid polyols;

b) adding to the thus obtained suspension a copper precursor and, optionally, minor amounts of other metal precursors;

c) heating the resulting suspension to a temperature of at least 60°C and keeping it under stirring at such temperature for a sufficient time to allow the reduction of said copper precursor of said and other optional metal precursors;

d) separating the solid phase obtained from the suspension obtained from the preceding step and washing the same with an organic solvent.

2. The process according to claim 1 wherein said polyol is ethylene glycol, pure or in admixture with other polyols.

3. The process according to claim 1 or claim 2, wherein said elemental tungsten powder has an average grain size in the range from 0.5 to 6 μm.

4. A process according to any one of claims 1-3, wherein said copper precursor is selected from the group consisting of cupric oxide (CuO), cuprous oxide (Cu_2O) and cuprous acetate monohydrate ($Cu(CH_3COO)_2.H_2O$).

5. A process according to any one of claims 2-4, wherein the heating of step *c)* is carried out at least at 70°C.

6. The process according to claim 5, wherein said step *c)* is carried out for a time between 4 and 6 hours.

7. A process according to any one of claims 2-4, wherein the heating of step *c)* is carried out at the boiling temperature of ethylene glycol (198°C) and said step is carried out for a time between 5 and 15 minutes.

8. A process according to any one of the preceding claims, wherein the tungsten-copper composite powder obtained from said step *d)* has a copper content of 15% by weight and a tungsten content of 85% by weight.

9. A process according to any one of claims 1-7, wherein in said step *b)*, besides said copper precursor, a minor amount of a cobalt (II) compound is also added.

10. The process according to claim 9, wherein the amount of said cobalt (II) compound is such that the resulting final composite powder has a content of cobalt metal of 0.5% by weight.

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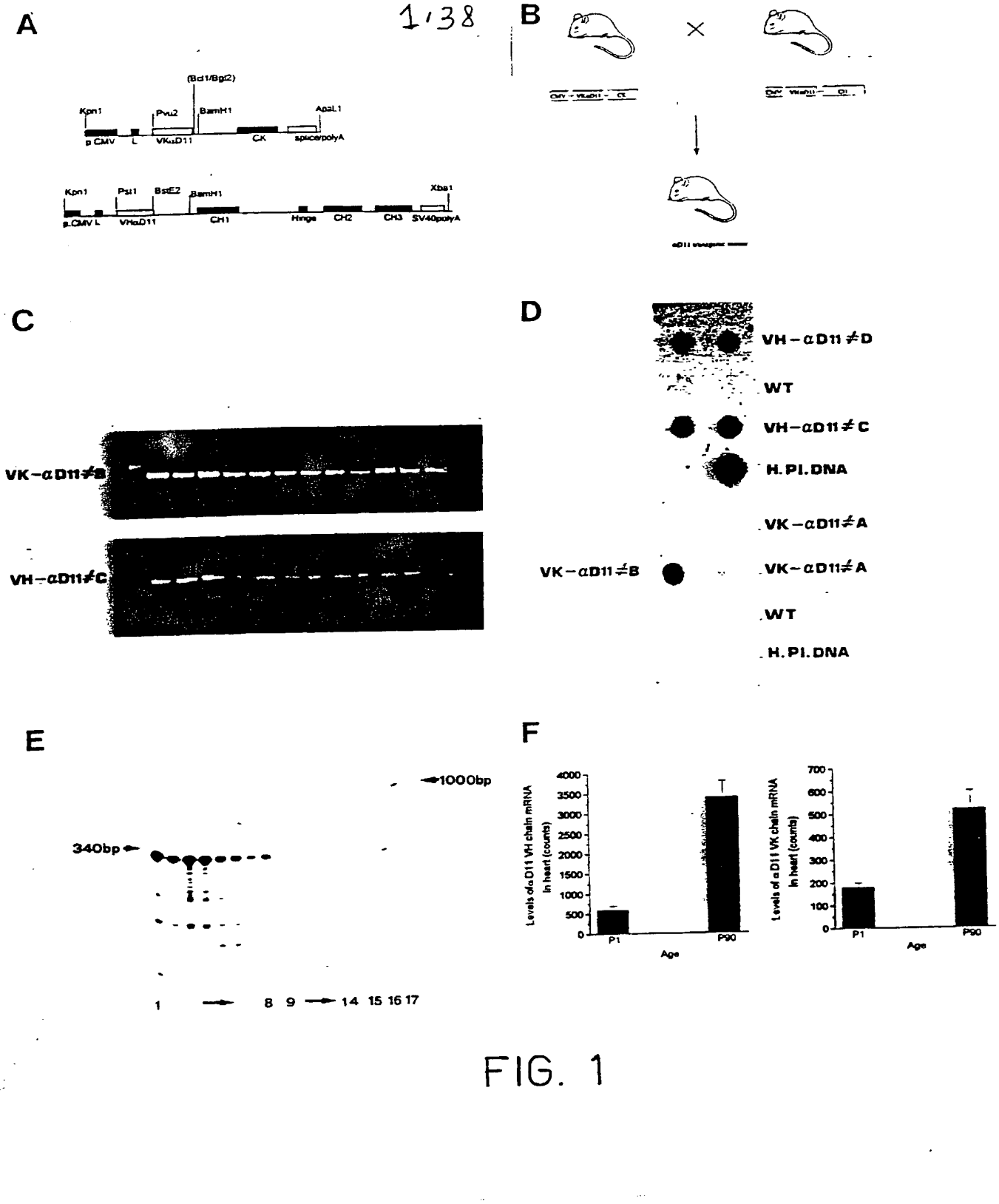
(54) Title: NON-HUMAN TRANSGENIC ANIMALS FOR THE STUDY OF NEURODEGENERATIVE SYNDROMES

(57) Abstract: Non-human transgenic animals as model study for human pathologies, being transgenic for an antibody are disclosed, in particular non-human transgenic animals for anti-NGF (Nerve Growth Factor) antibody able to mimic different pathologies, as neurodegenerative syndromes, to be used as a model to study said pathologies and provide therapies therefor.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Kpn1
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Pvu2
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Pst1
BstE2
BamH1
Xba1
p.CMV L
VHαD11
CH1
Hinge
CH2
CH3
SV40polyA
B
C
VK−αD11≠B
VH−αD11≠C
D
VH−αD11≠D
WT
VH−αD11≠C
H.PI.DNA
VK−αD11≠A
VK−αD11≠B
VK−αD11≠A
WT
H.PI.DNA
E
1000bp
340bp
1
8 9
14 15 16 17
F
Levels of αD11 VH chain mRNA in heart (counts)
4000
3500
3000
2500
2000
1500
1000
500
0
P1
P90
Age
Levels of αD11 VK chain mRNA in heart (counts)
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600
500
400
300
200
100
0
P1
P90
Age

FIG. 1

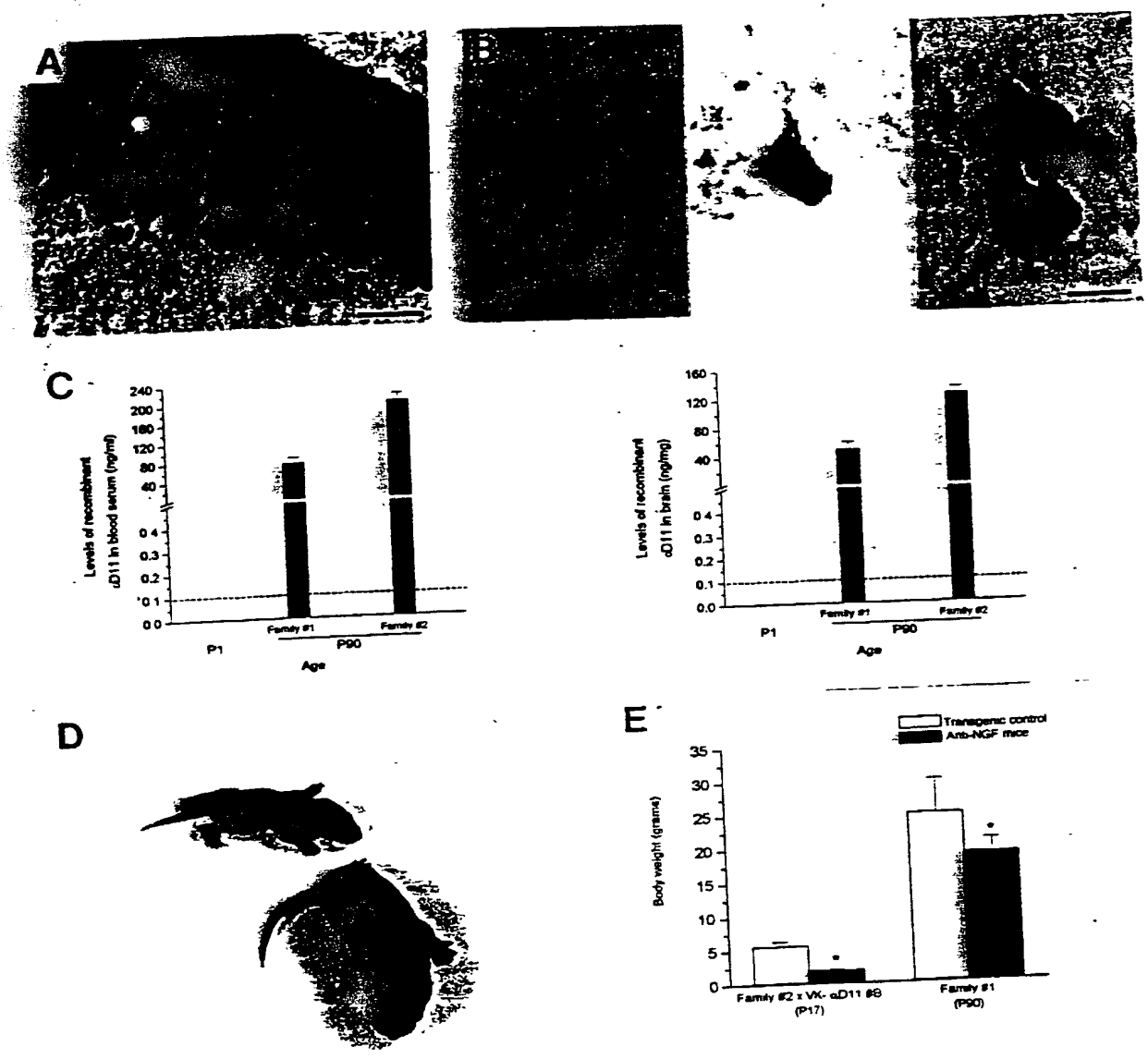

A
B
C
Levels of recombinant αD11 in blood serum (ng/ml)
240
200
160
120
80
40
0.4
0.3
0.2
0.1
0.0
Family #1
Family #2
P1
P90
Age
Levels of recombinant αD11 in brain (ng/mg)
160
120
80
40
0.4
0.3
0.2
0.1
0.0
Family #1
Family #2
P1
P90
Age
D
E
Transgenic control
Anti-NGF mice
Body weight (grams)
35
30
25
20
15
10
5
0
*
*
Family #2 x VK- αD11 #8
(P17)
Family #1
(P90)

FIG. 2

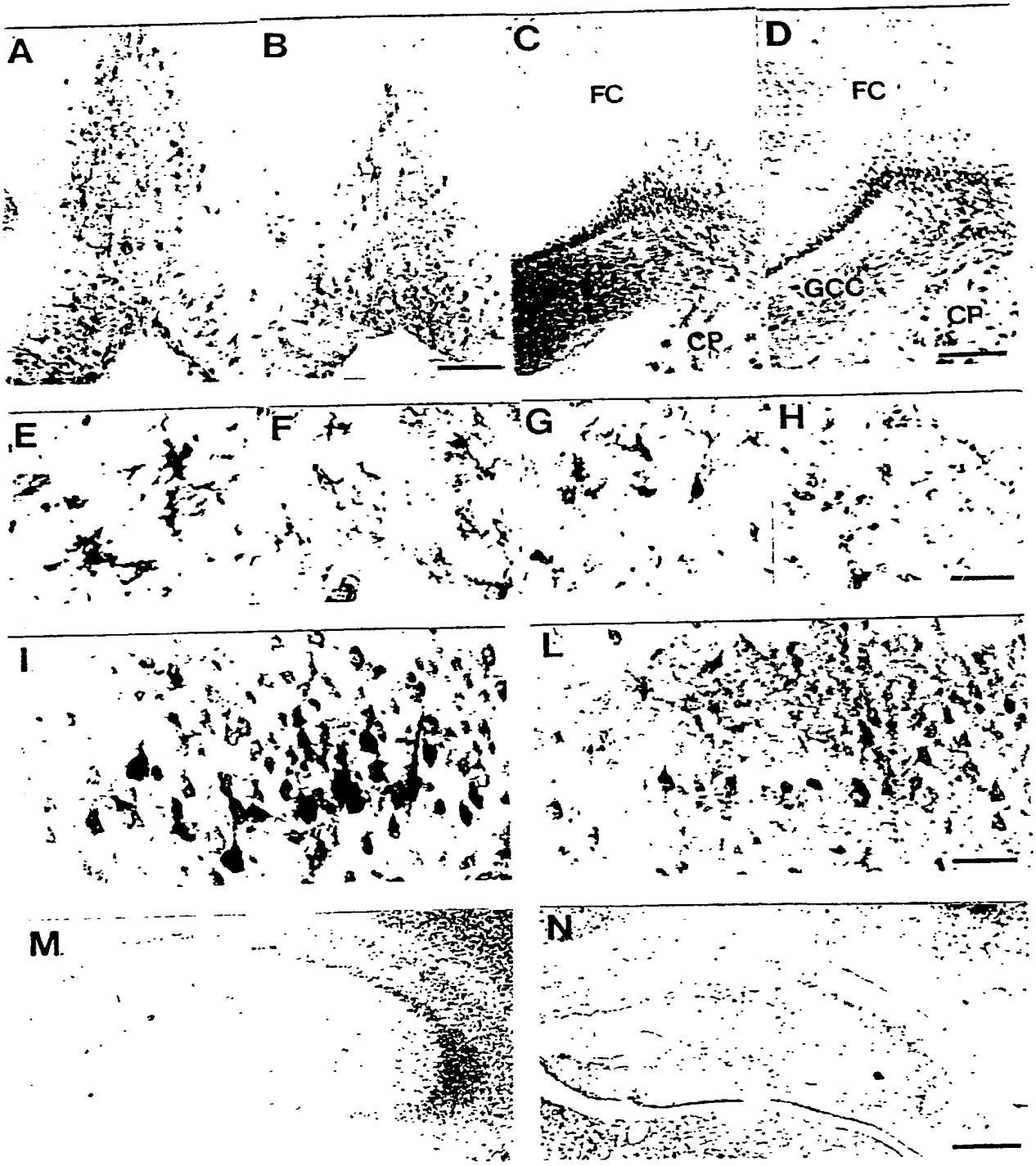


FIG. 3

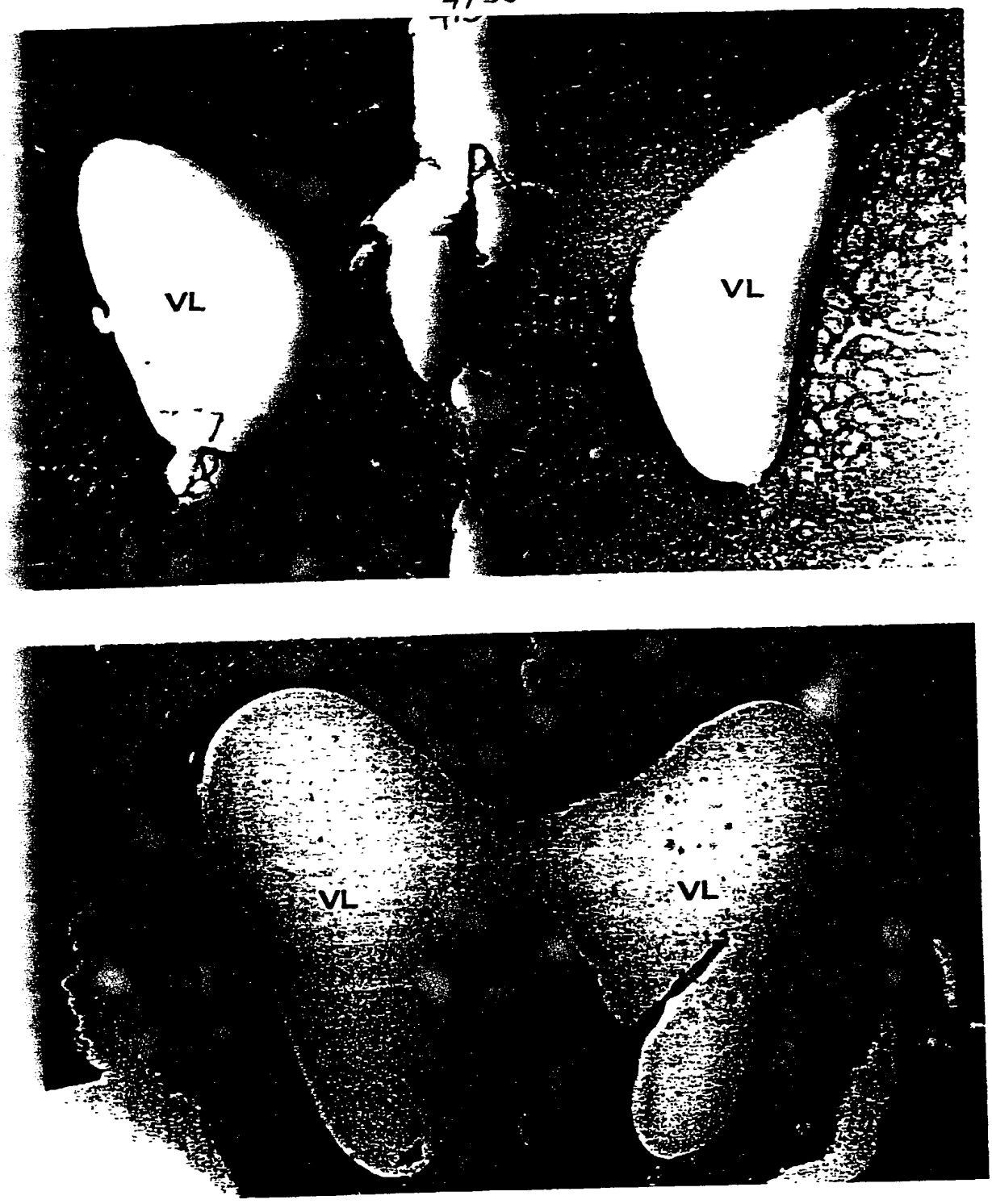
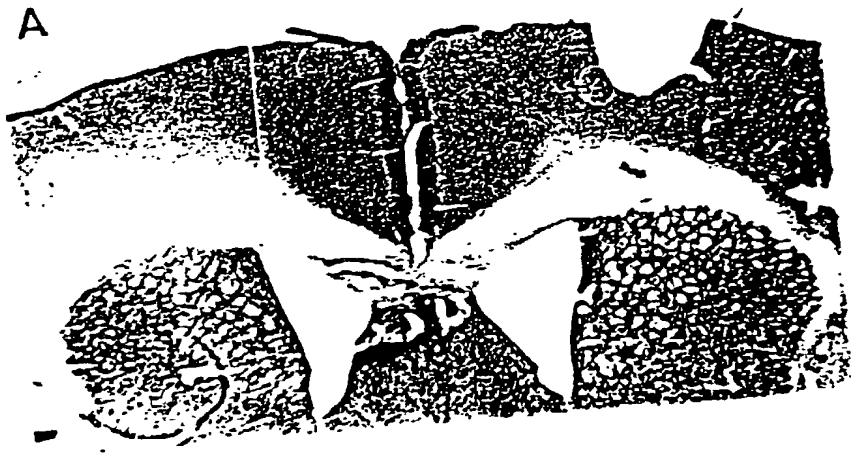


FIG. 4

A



B

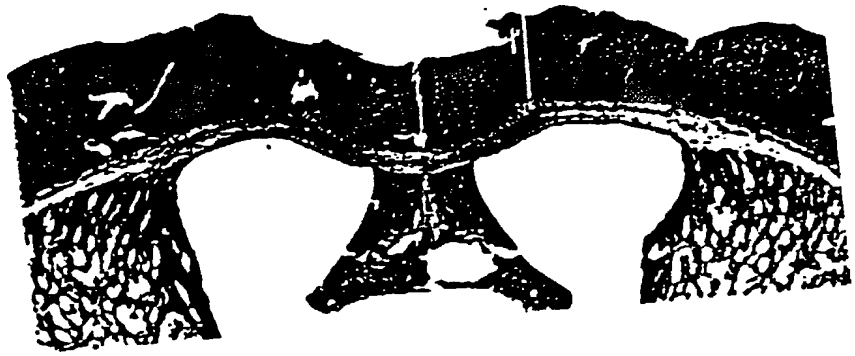
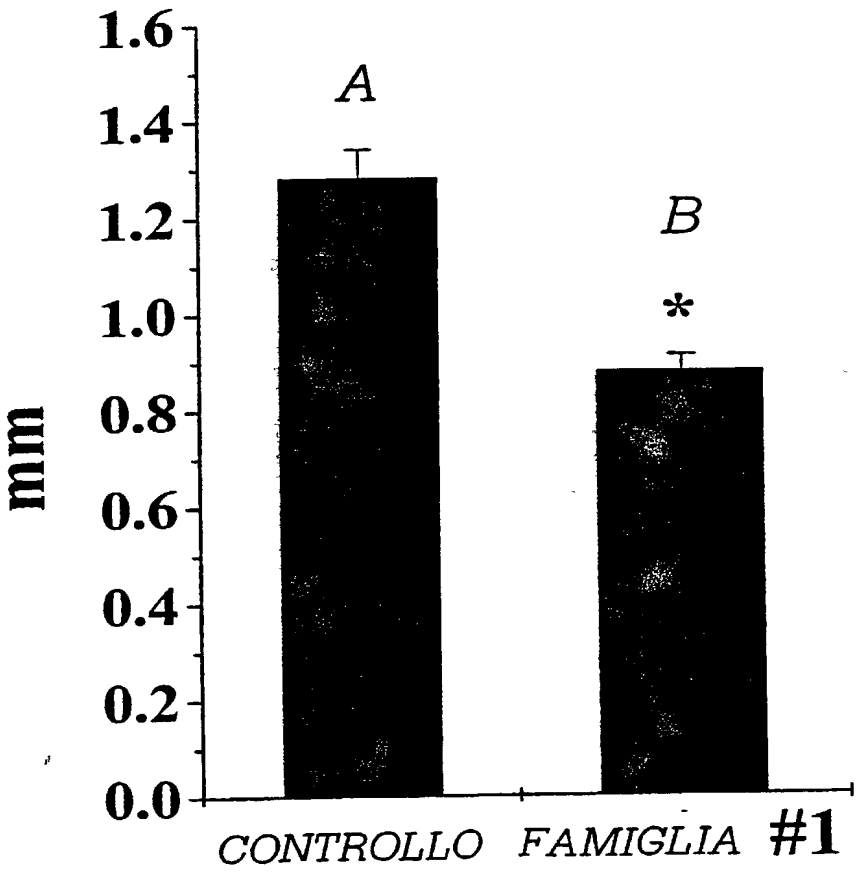


FIG. 5

SPESSORE CORTICALE



CONTROLLO : 1.282 +/- 0.059

FAMIGLIA #1: 0.879+/- 0.033

FIG. 5c

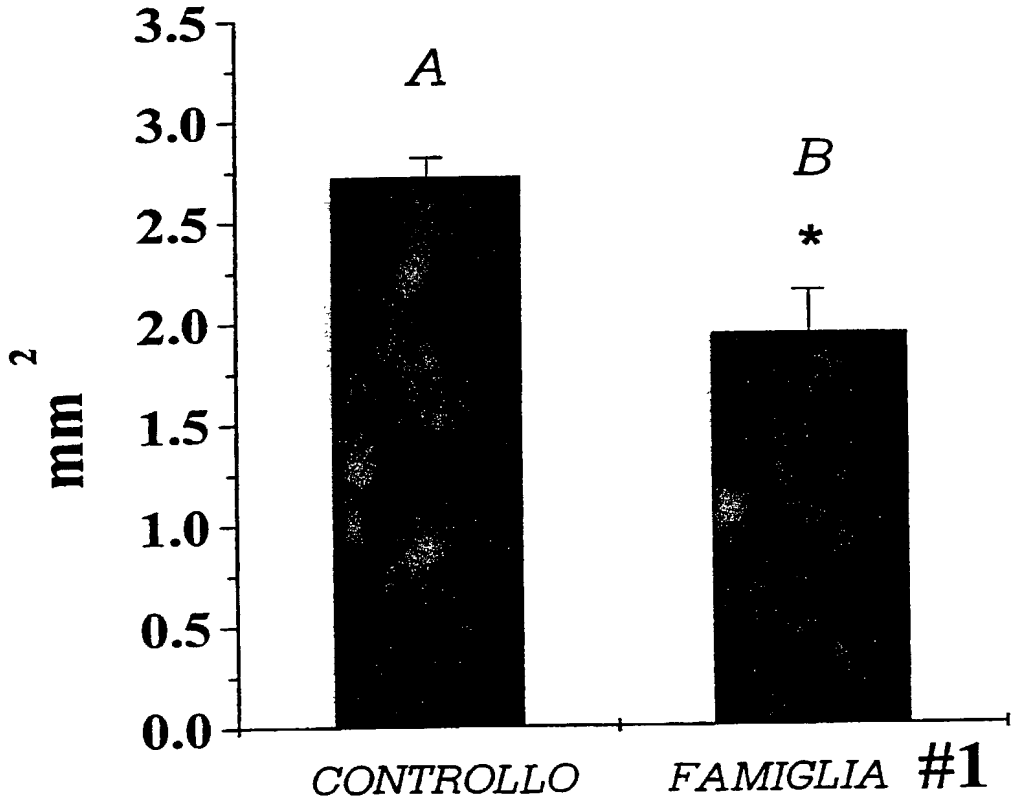
7138

A

B

FIG. 6

SUPERFICIE DELL IPPOCAMPO



CONTROLLO : 2.72 +/- 0.098

FAMIGLIA #1: 1.94 +/- 0.211

FIG. 6c

A

B

FIG. 7

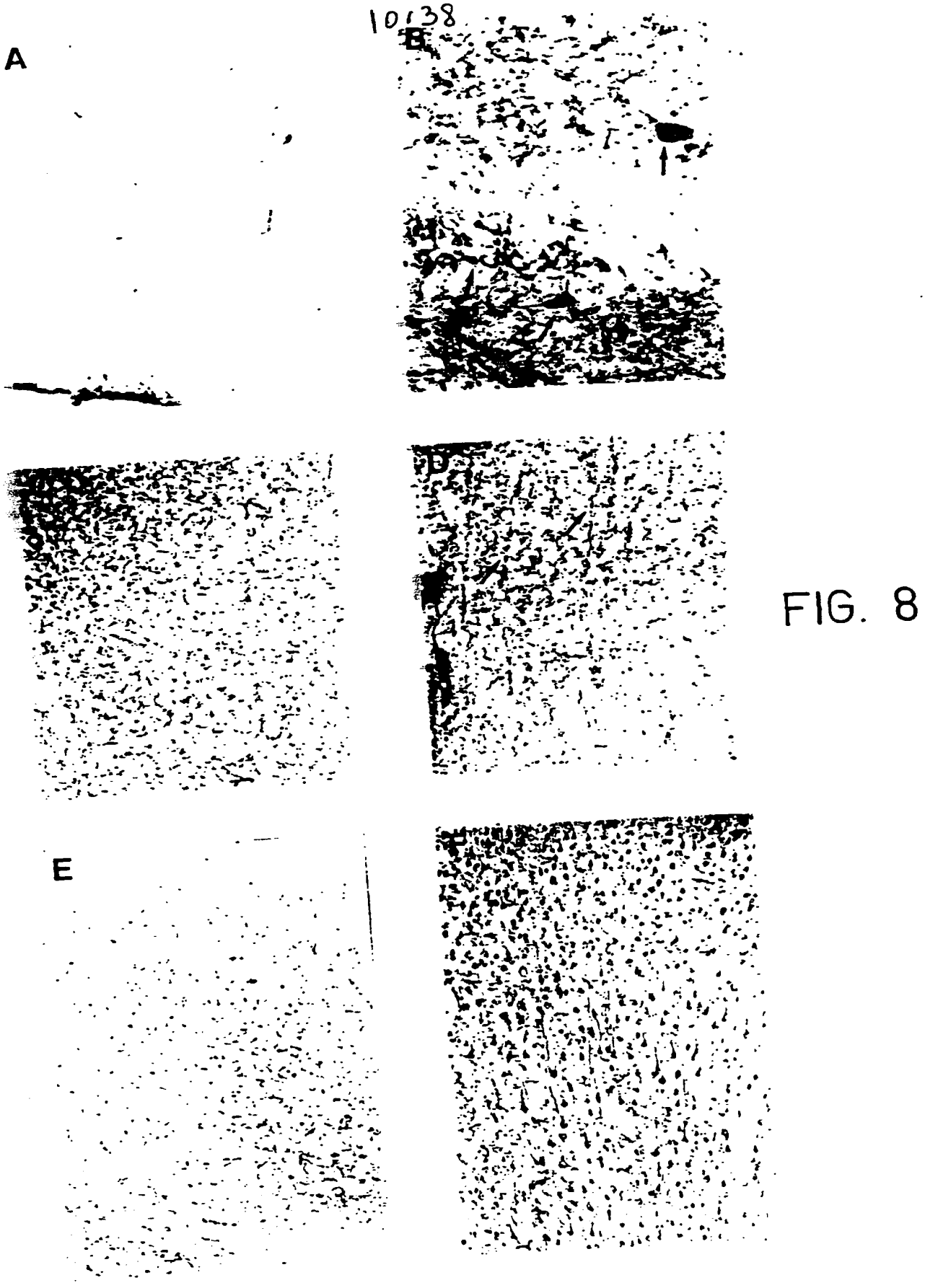


FIG. 8

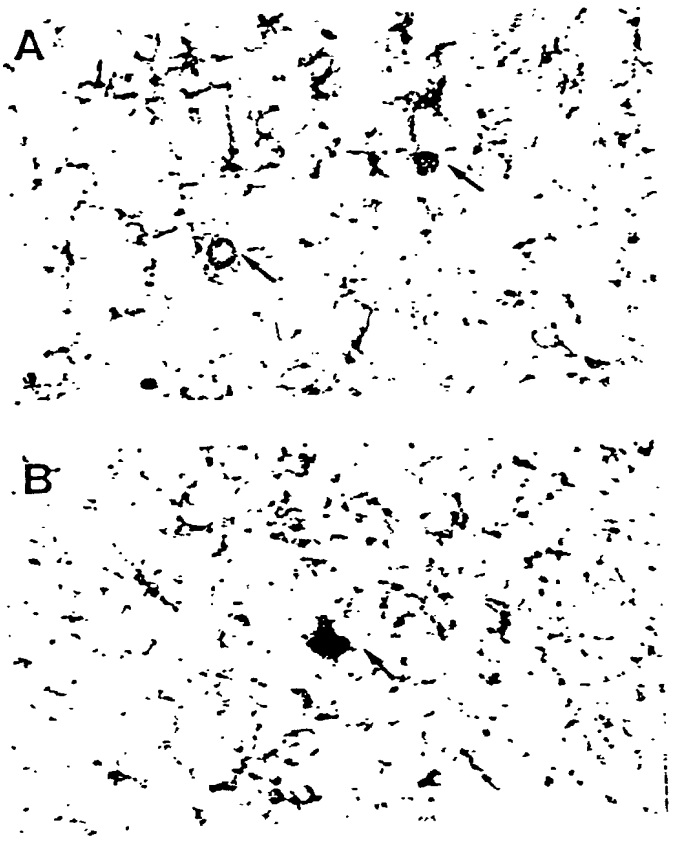


FIG. 9

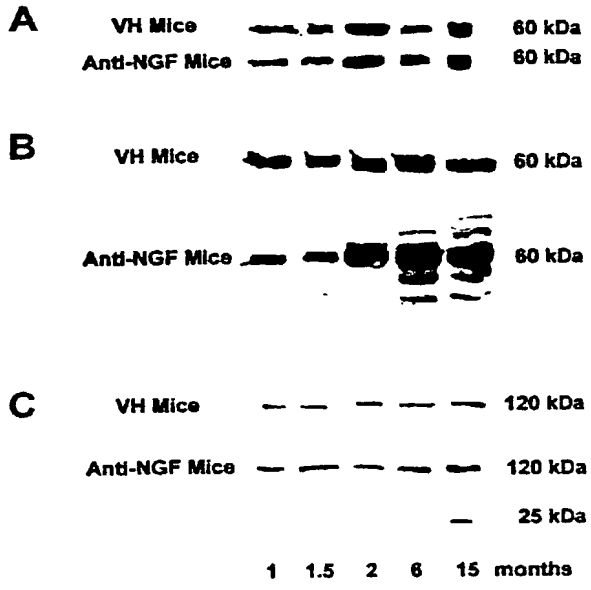

A
VH Mice
60 kDa
Anti-NGF Mice
60 kDa
B
VH Mice
60 kDa
Anti-NGF Mice
60 kDa
C
VH Mice
120 kDa
Anti-NGF Mice
120 kDa
25 kDa
1 1.5 2 6 15 months

FIG. 10

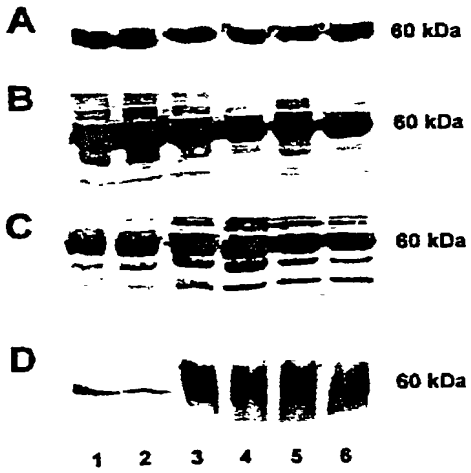


FIG. 11

14138

FIG. 12

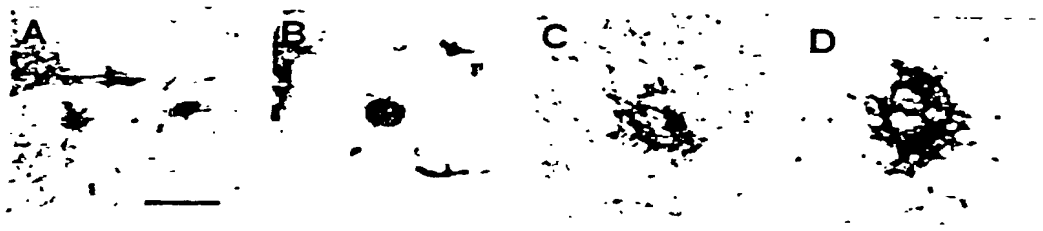


FIG. 13

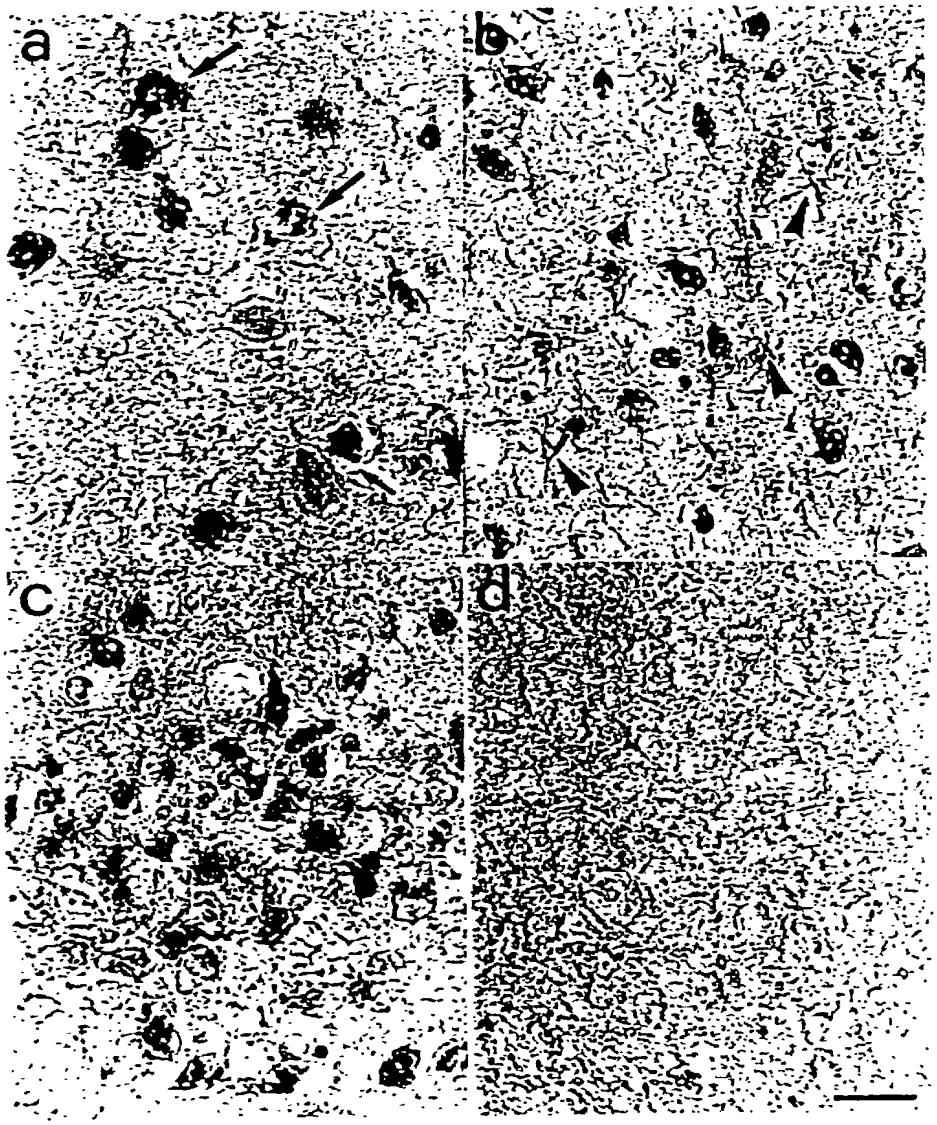


FIG. 14

17138

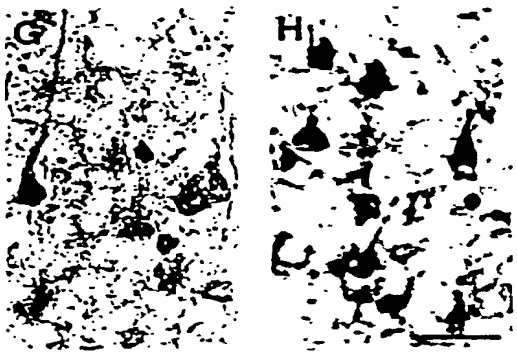


FIG. 15

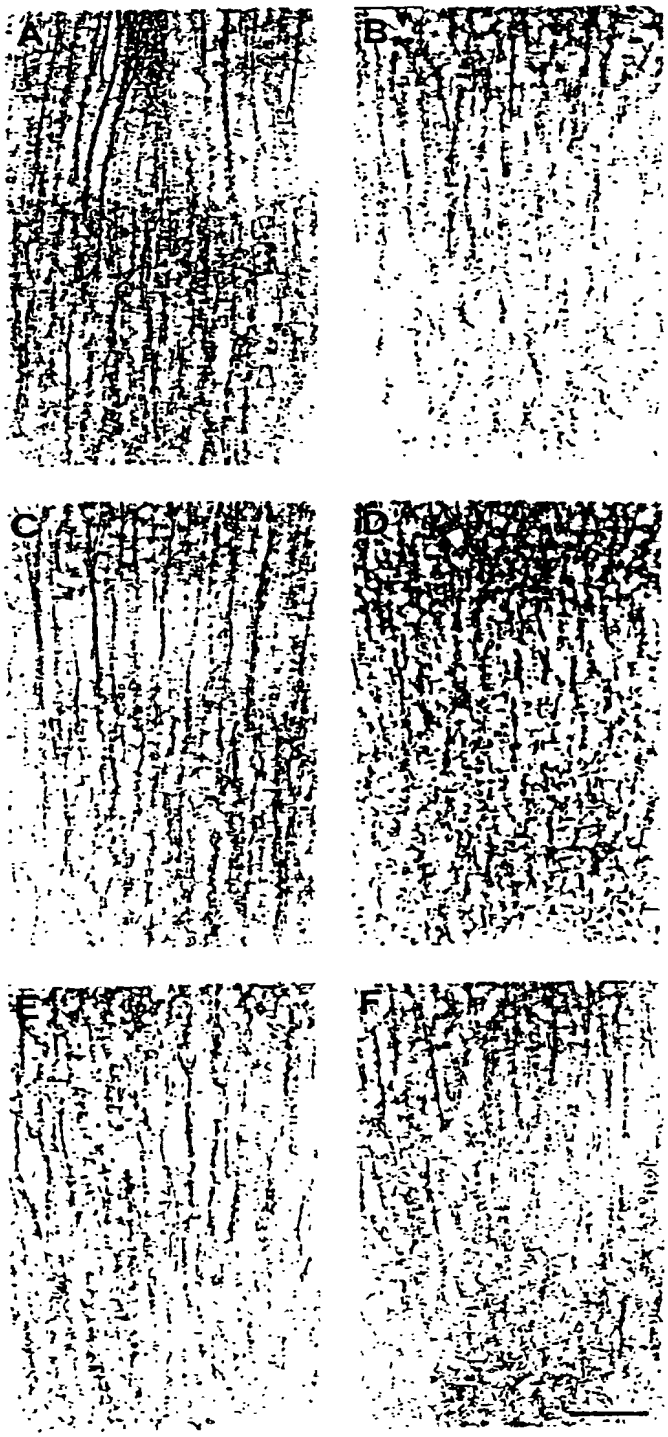


FIG. 16

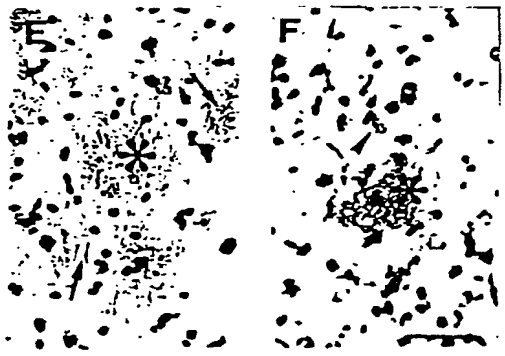


FIG. 17

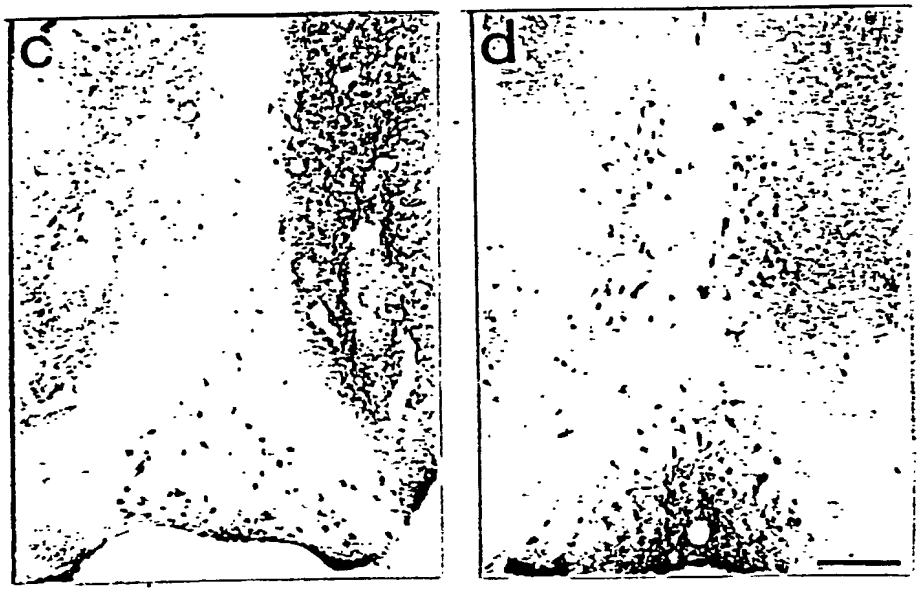


FIG. 18

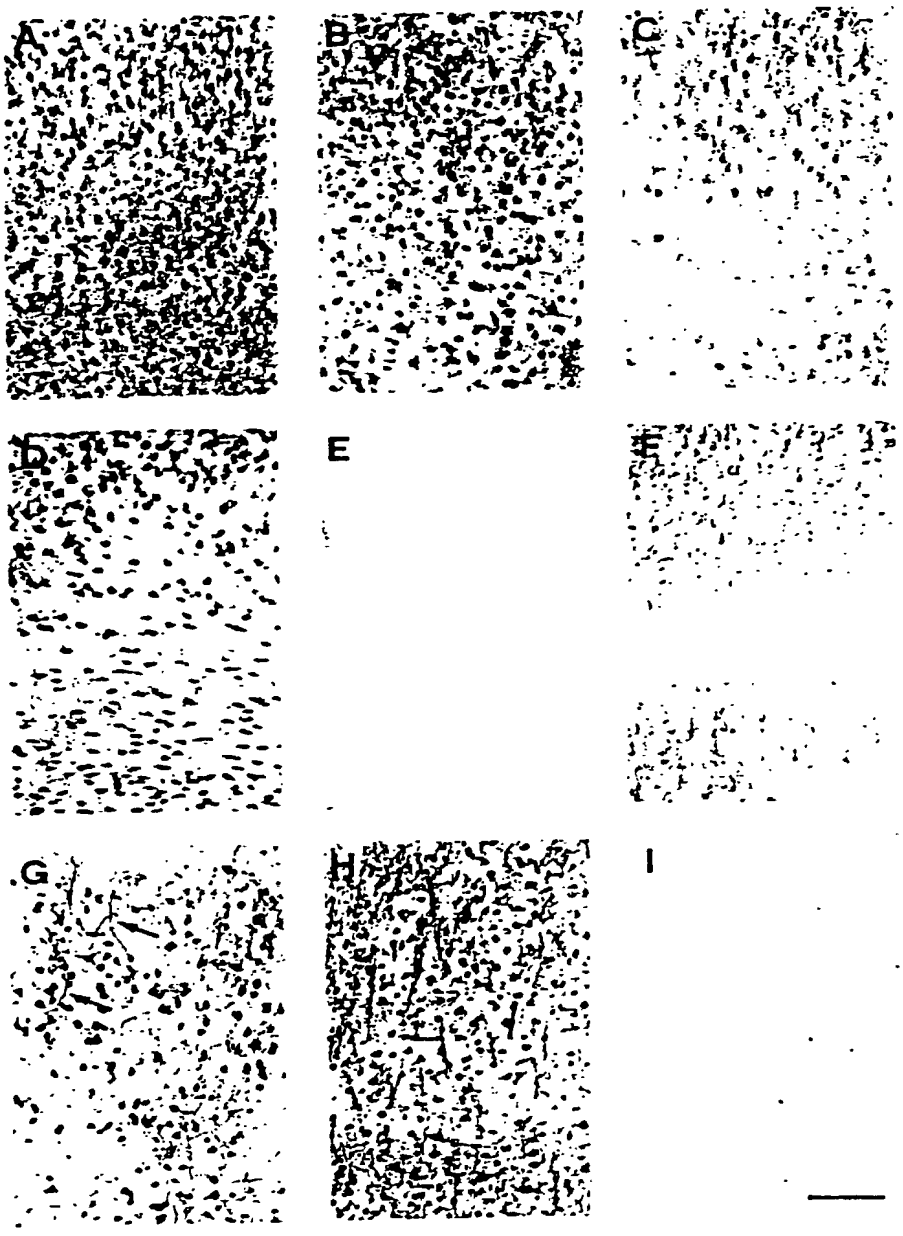


FIG. 19

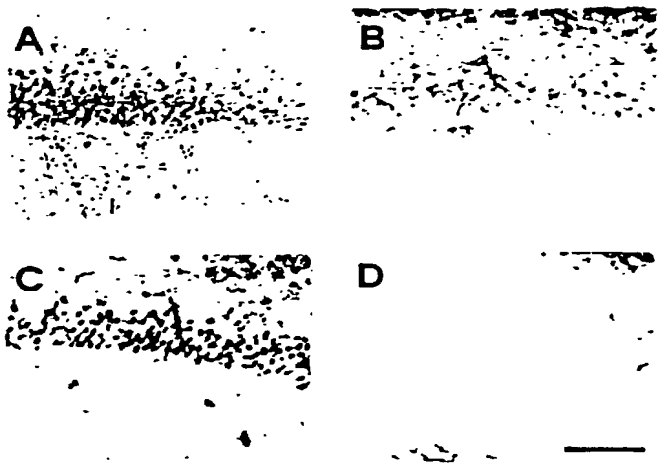


FIG. 20

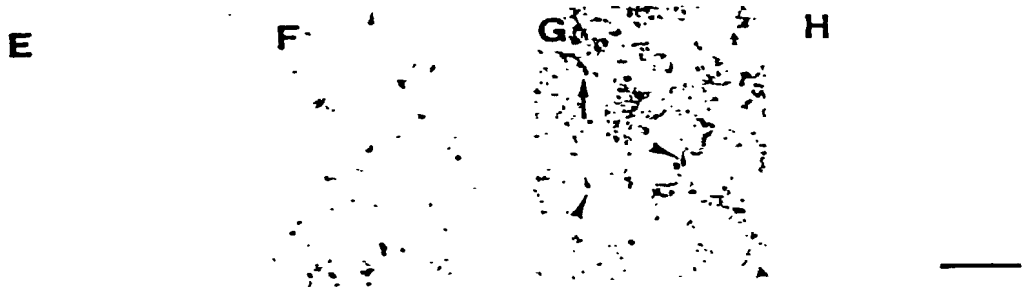


FIG. 21

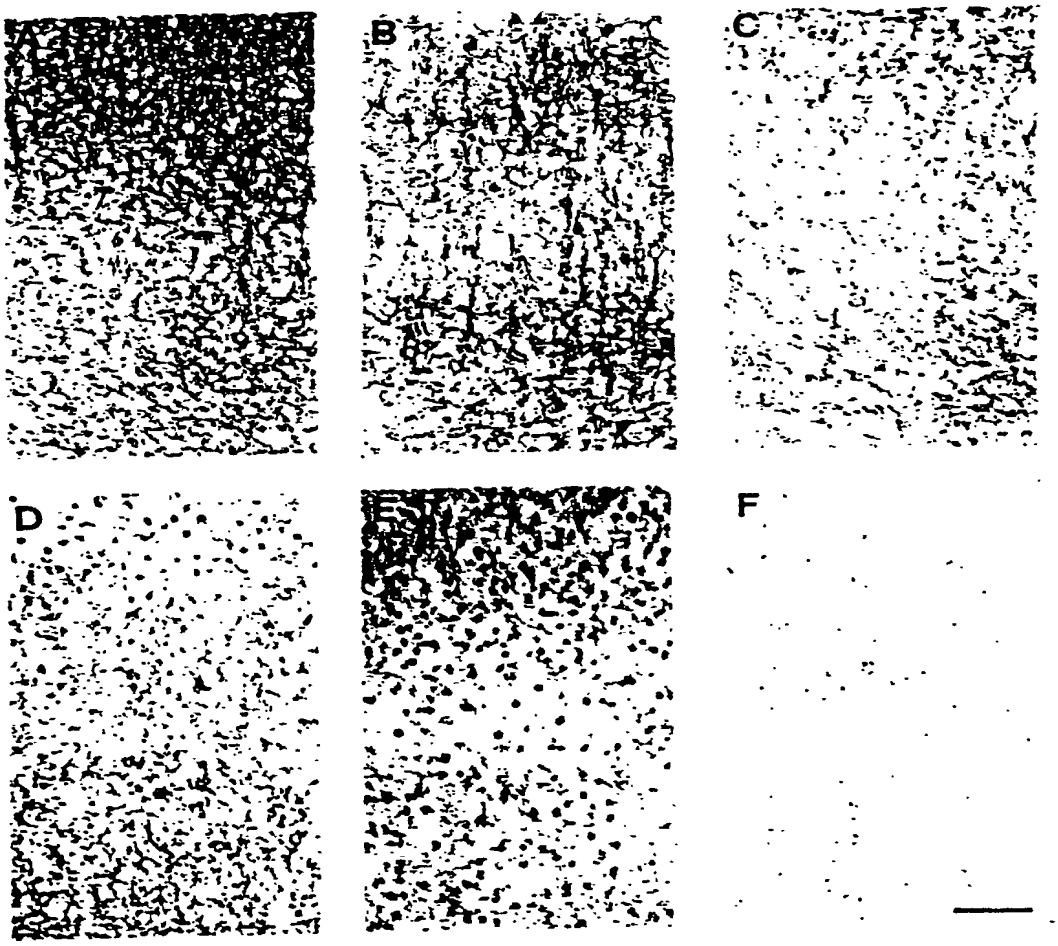


FIG. 22

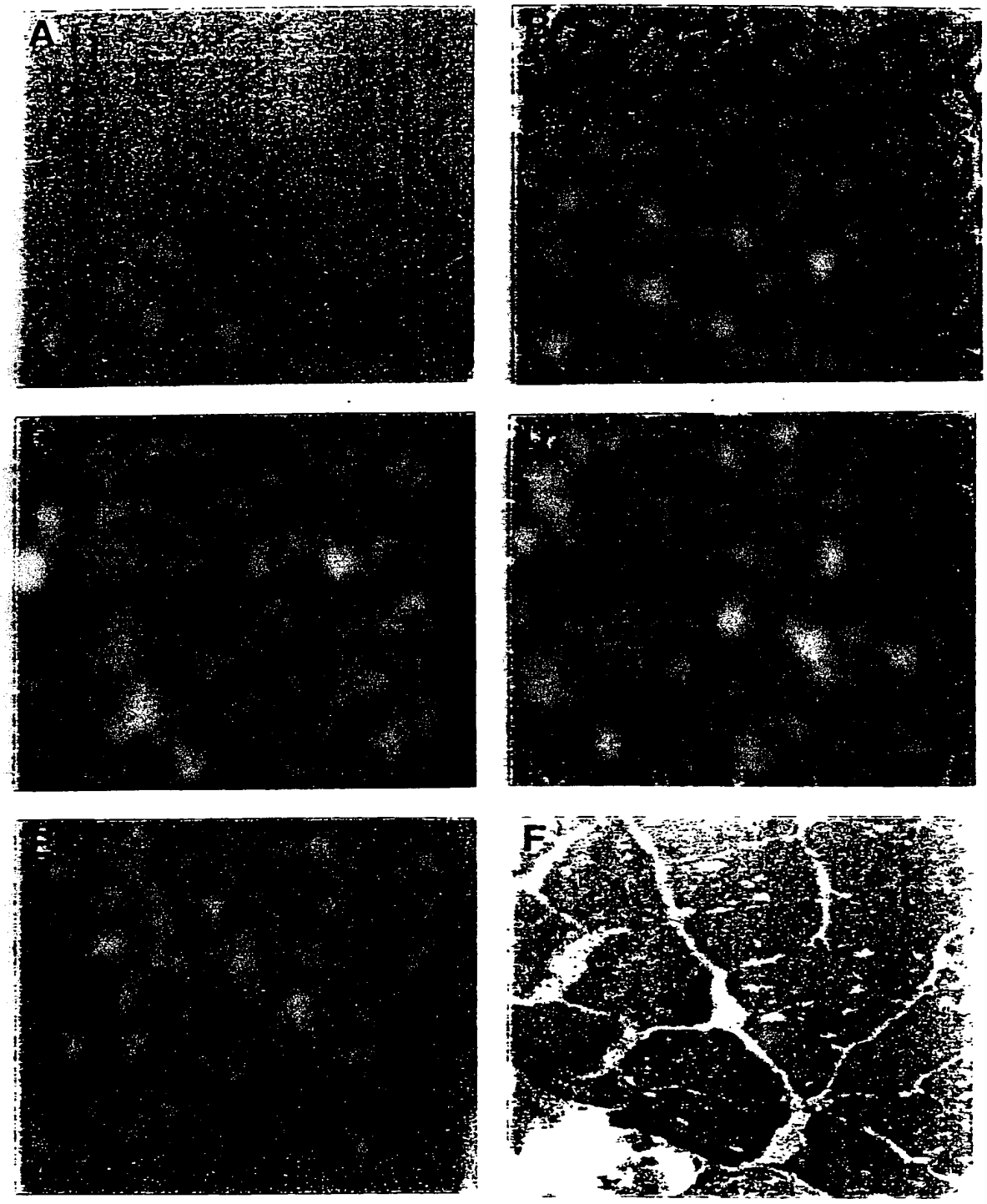


FIG. 23

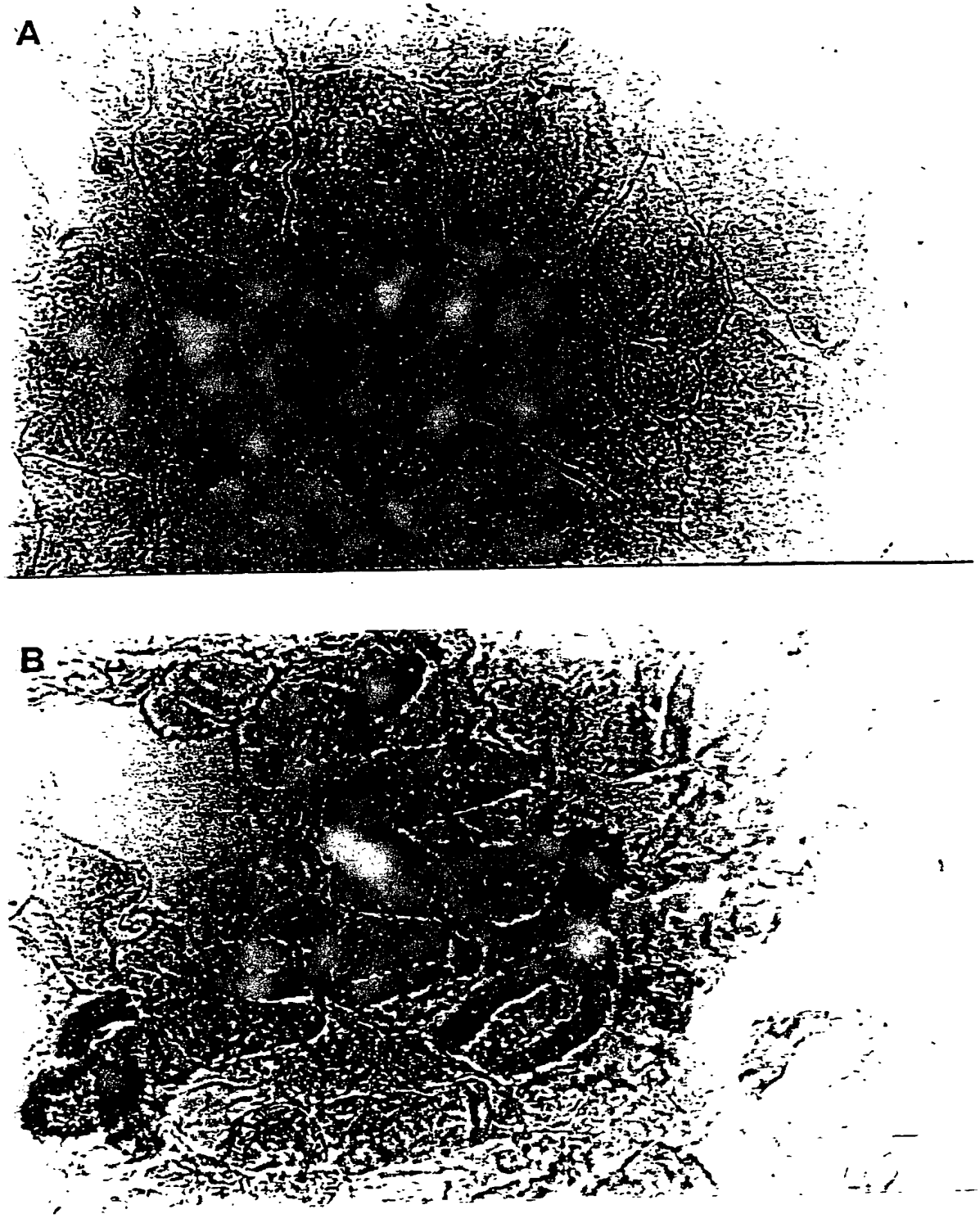


FIG. 24

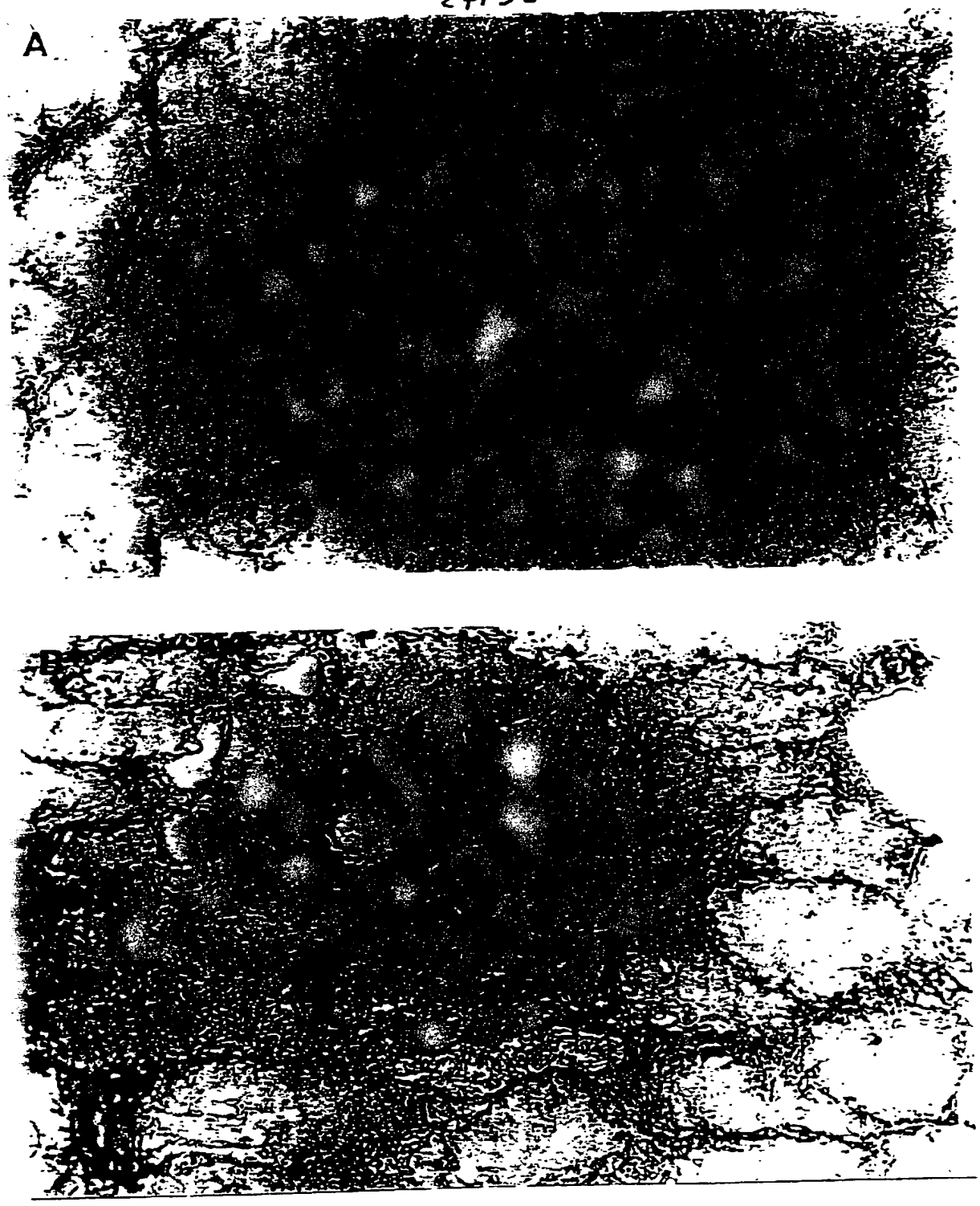


FIG. 25

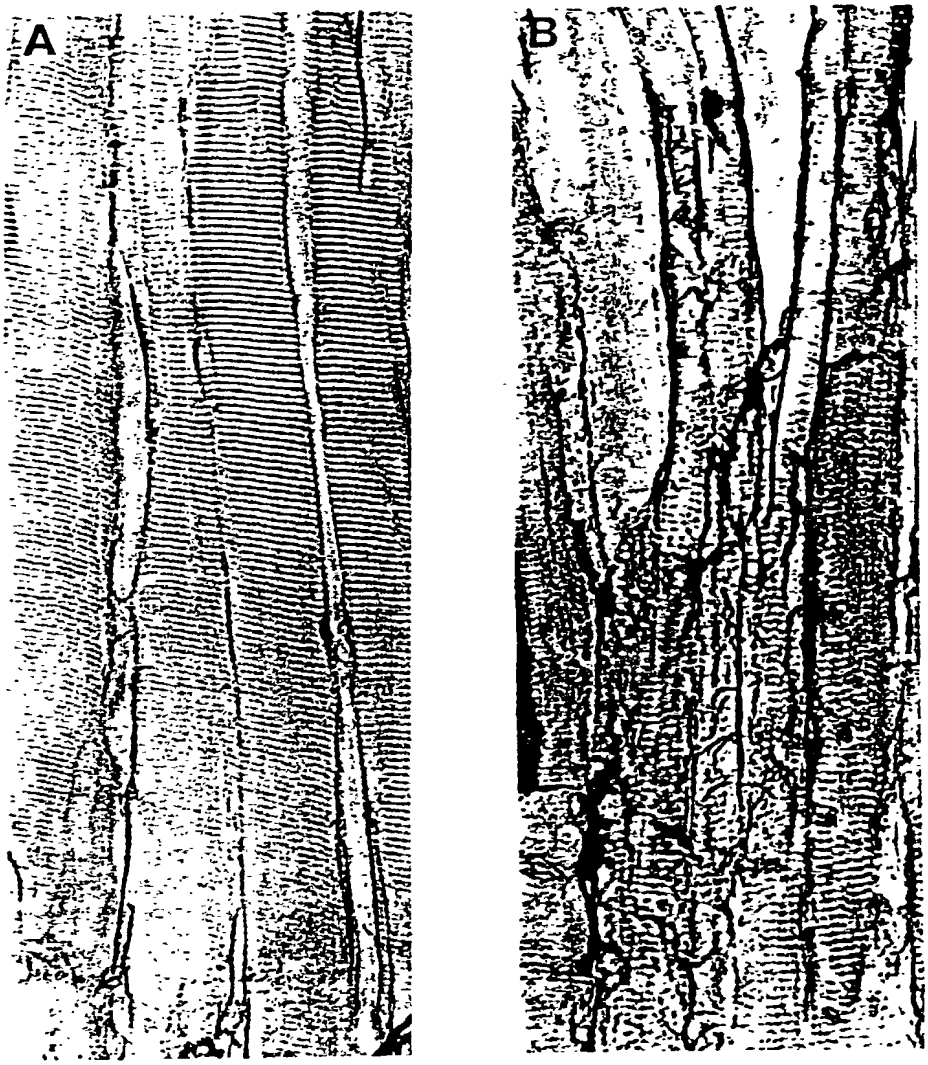


FIG. 26

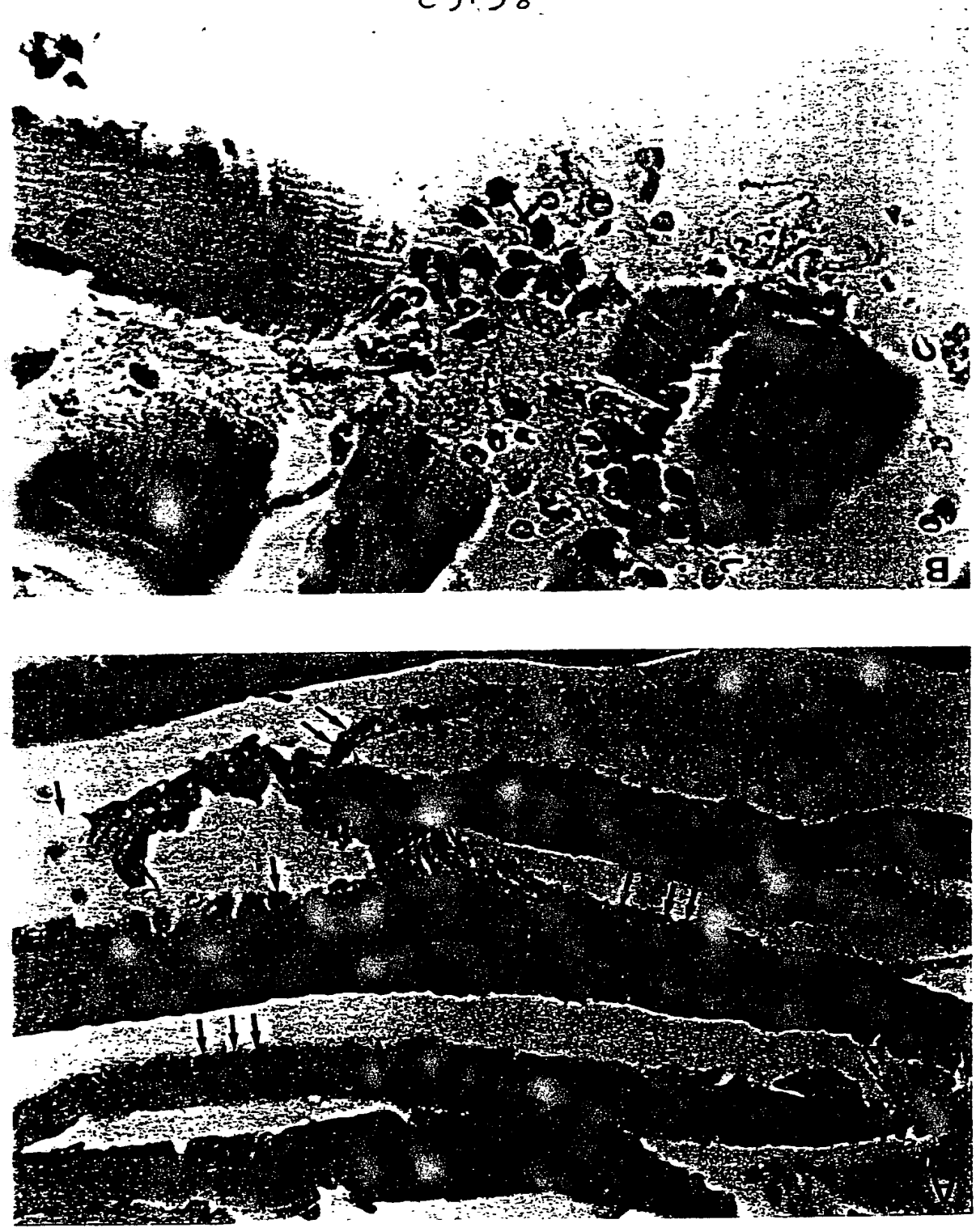


FIG. 27

FIG. 28

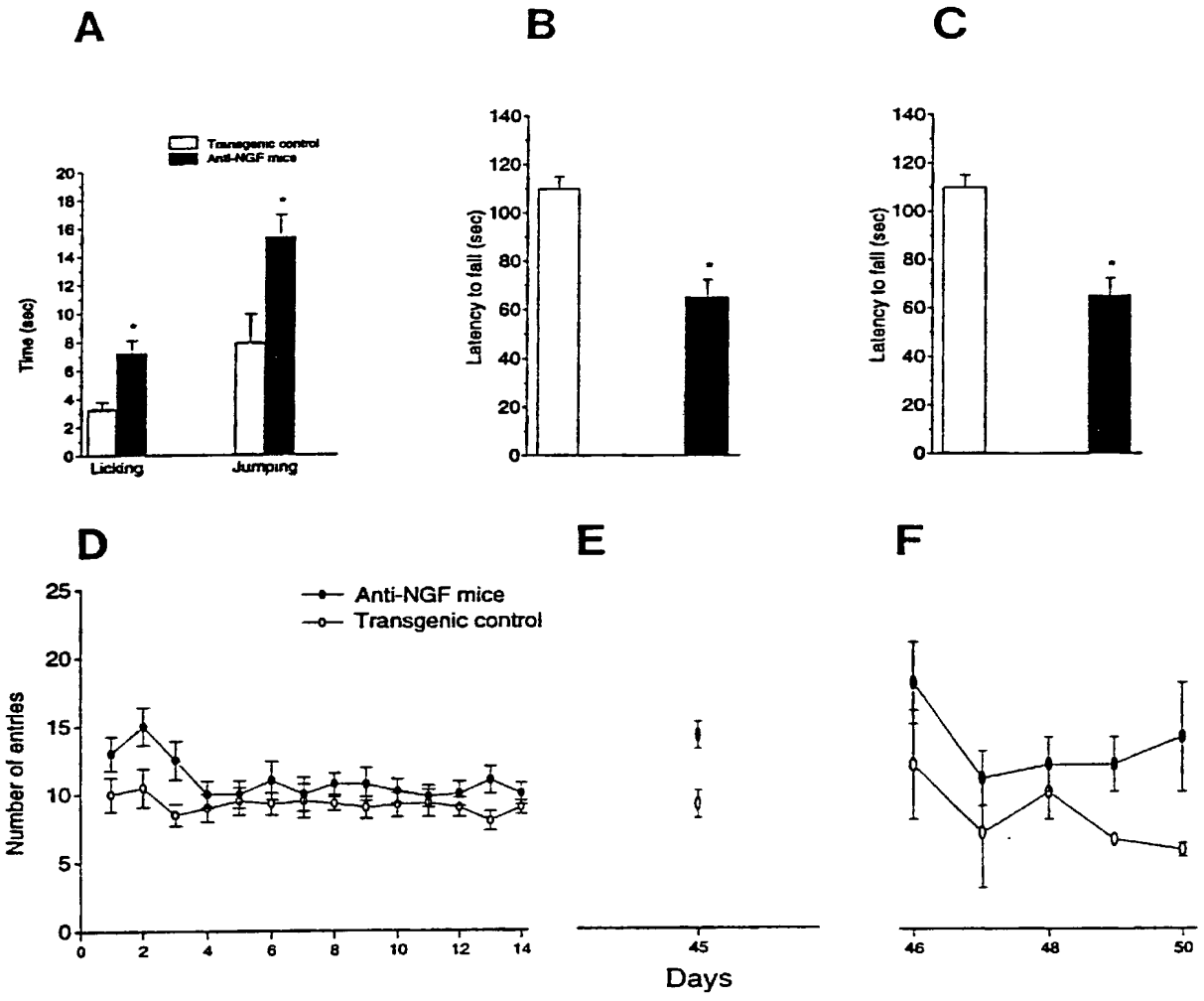

A
B
C
Transgenic control
Anti-NGF mice
Time (sec)
Licking
Jumping
Latency to fall (sec)
Latency to fall (sec)
D
E
F
Anti-NGF mice
Transgenic control
Number of entries
Days

FIG 29

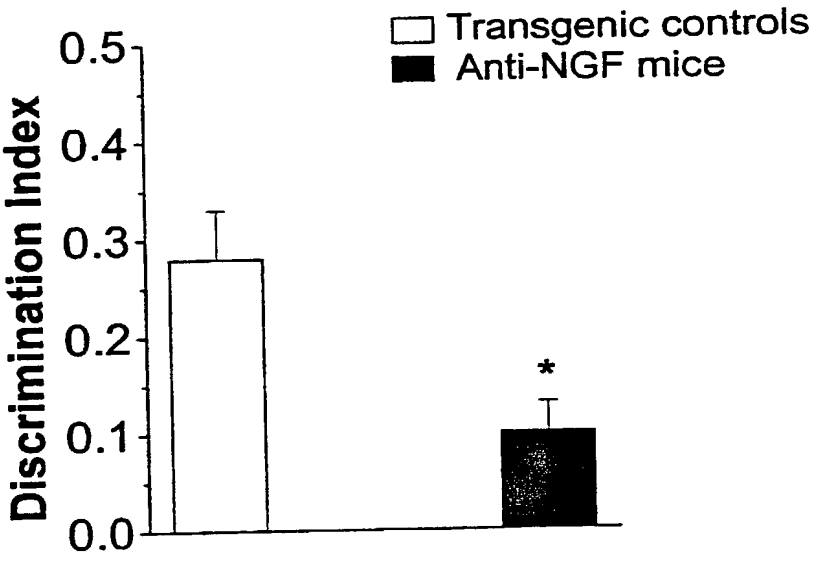

Transgenic controls
Anti-NGF mice
Discrimination Index
0.5
0.4
0.3
0.2
0.1
0.0
*

FIG. 30

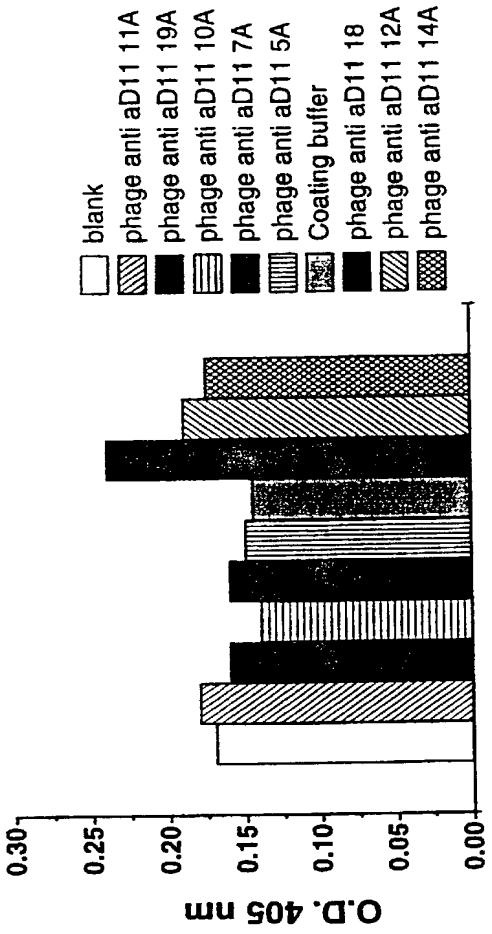

blank
phage anti aD11 11A
phage anti aD11 19A
phage anti aD11 10A
phage anti aD11 7A
phage anti aD11 5A
Coating buffer
phage anti aD11 18
phage anti aD11 12A
phage anti aD11 14A
0.30
0.25
0.20
0.15
0.10
0.05
0.00
O.D. 405 nm

FIG 31

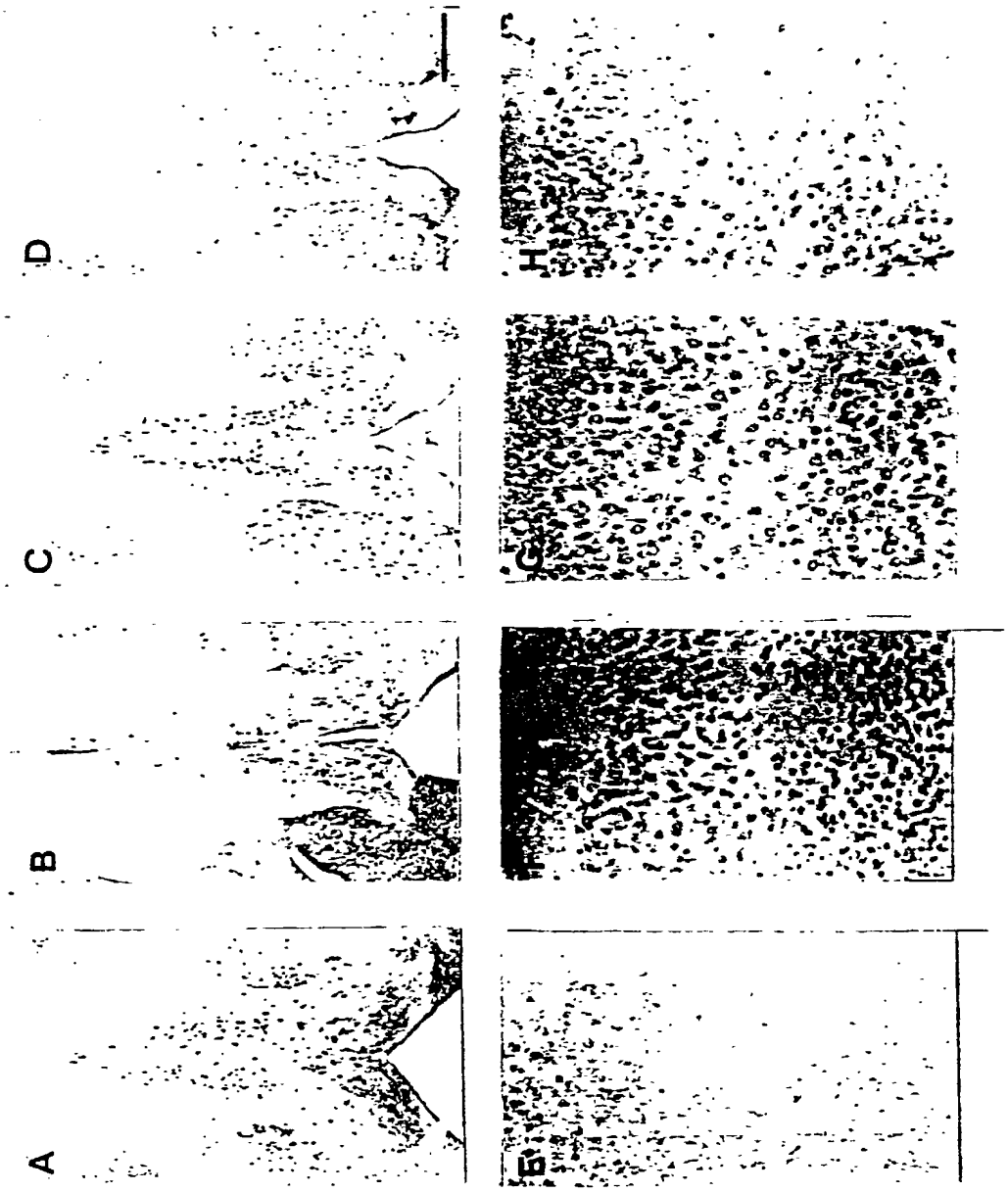


FIG 32

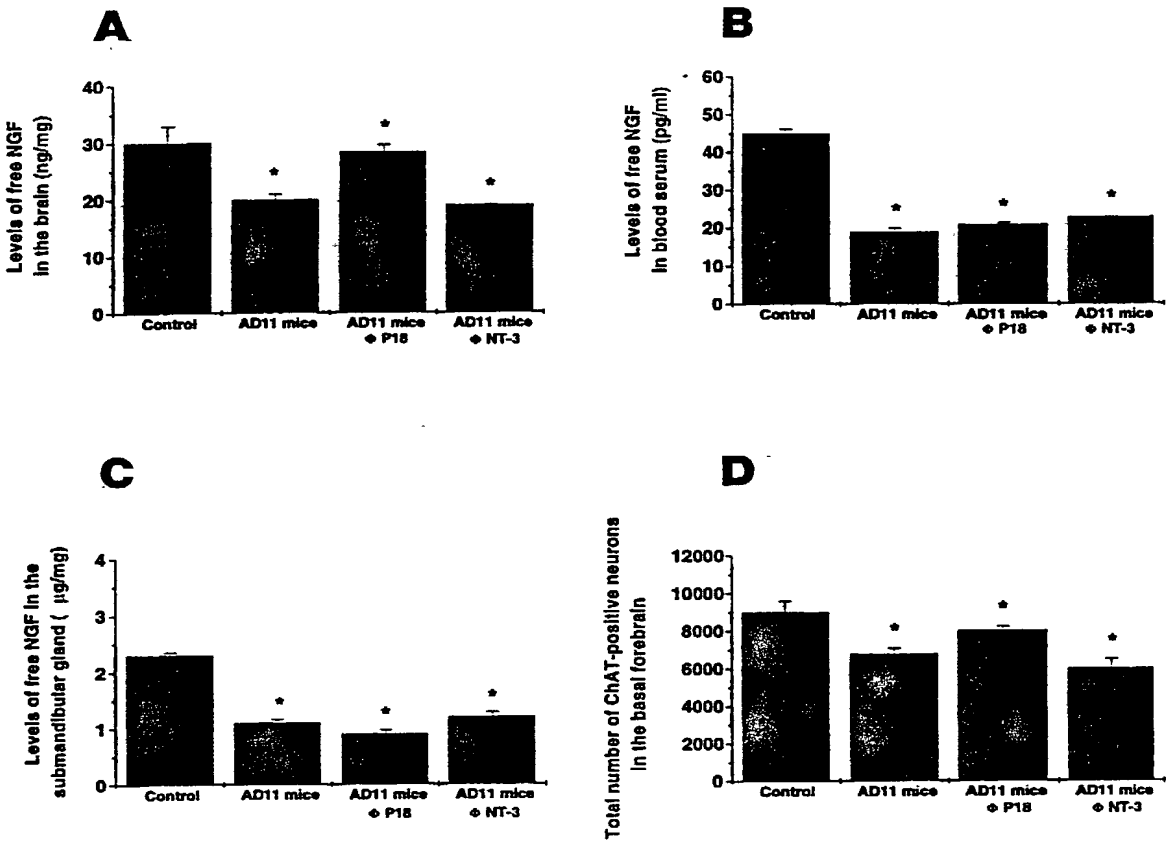

A
Levels of free NGF in the brain (ng/mg)
40
30
20
10
0
Control
AD11 mice
AD11 mice + P18
AD11 mice + NT-3
B
Levels of free NGF in blood serum (pg/ml)
60
50
40
30
20
10
0
Control
AD11 mice
AD11 mice + P18
AD11 mice + NT-3
C
Levels of free NGF in the submandibular gland (µg/mg)
4
3
2
1
0
Control
AD11 mice
AD11 mice + P18
AD11 mice + NT-3
D
Total number of ChAT-positive neurons in the basal forebrain
12000
10000
8000
6000
4000
2000
0
Control
AD11 mice
AD11 mice + P18
AD11 mice + NT-3

FIG 33

36/38

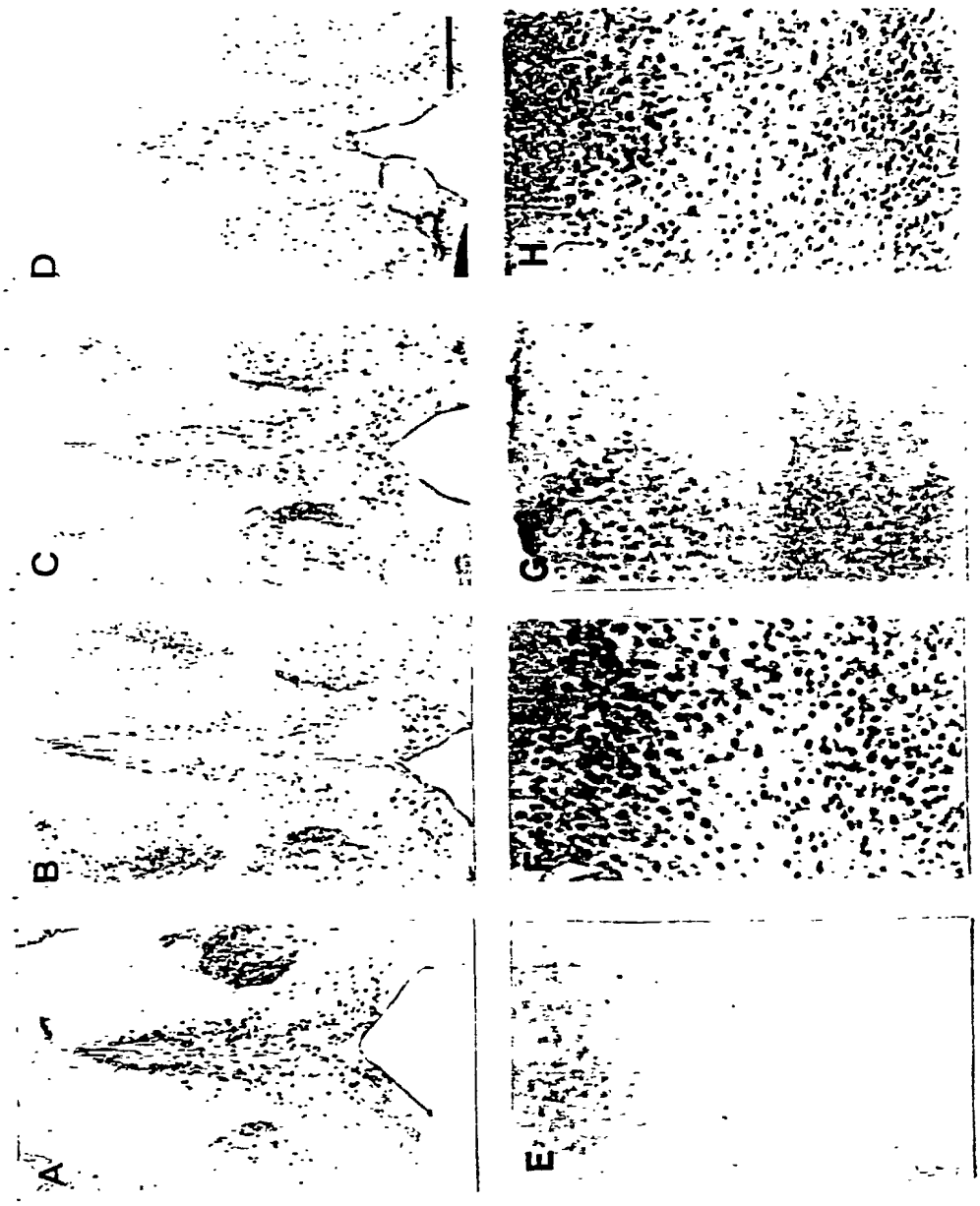


FIG 34

37/38

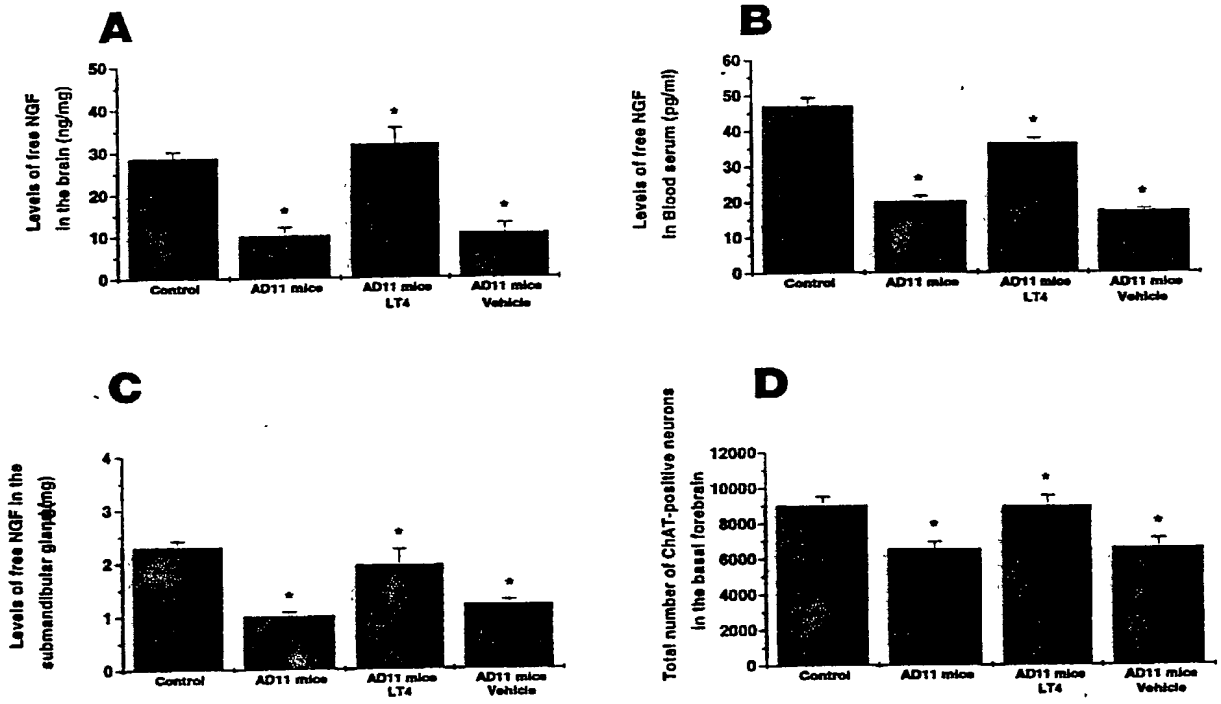

A
Levels of free NGF in the brain (ng/mg)
50
40
30
20
10
0
Control
AD11 mice
AD11 mice LT4
AD11 mice Vehicle
B
Levels of free NGF in Blood serum (pg/ml)
60
50
40
30
20
10
0
Control
AD11 mice
AD11 mice LT4
AD11 mice Vehicle
C
Levels of free NGF in the submandibular gland(mg)
4
3
2
1
0
Control
AD11 mice
AD11 mice LT4
AD11 mice Vehicle
D
Total number of ChAT-positive neurons in the basal forebrain
12000
10000
8000
6000
4000
2000
0
Control
AD11 mice
AD11 mice LT4
AD11 mice Vehicle

FIG 35

10/049306
PCT/IT00/00321

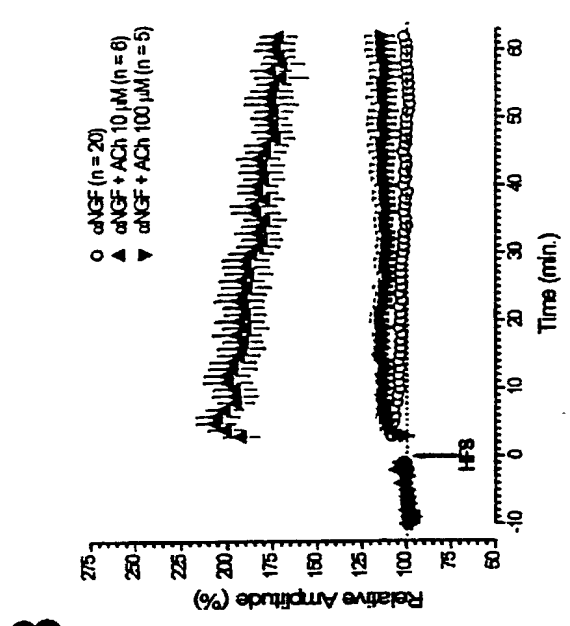
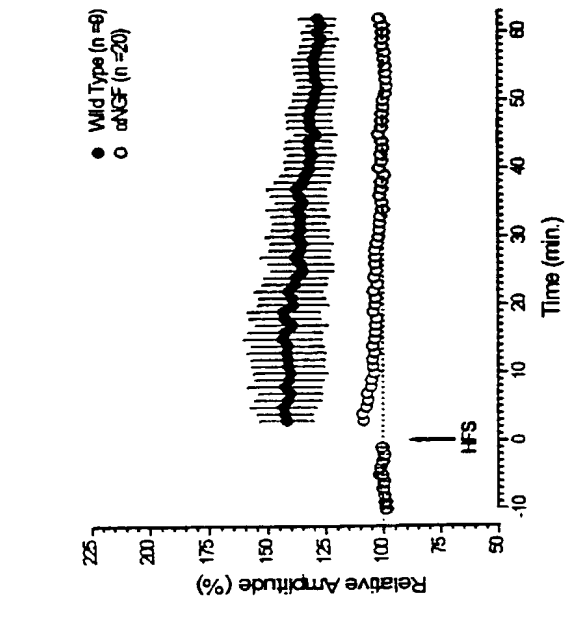
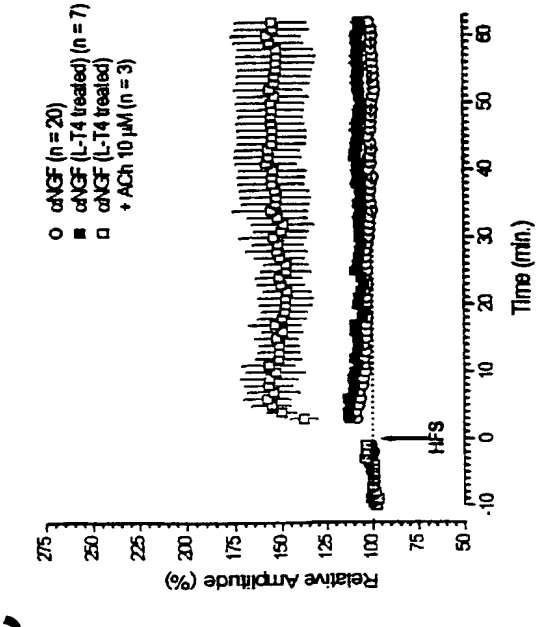


FIG 36





DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

MODULO DI DICHIARAZIONE PER DOMANDA DI BREVETTO

ITALIAN LANGUAGE DECLARATION

Io, sottoscritto inventore, dichiaro con il presente che:

As a below named inventor, I hereby declare that:

Il mio domicilio, recapito postale e cittadinanza sono quelli indicati in calce accanto al mio nome.

My residence, post office address and citizenship are as stated below next to my name.

Che mi reputo in buona fede essere l'inventore originario, primo e unico (qualora un solo nominativo appaia elencato appresso) o il coinventore (qualora i nominativi siano più di uno) primo e originario dell'invenzione da me rivendicata, e per la quale faccio domanda di brevetto Tale invenzione è chiamata:

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed nd for which a patent is sought on the invention entitled:

NON-HUMAN TRANSGENIC ANIMALS FOR THE STUDY OF NEURODEGENERATIVE SYNDROMES

E la sua descrizione è allegata alla presente Dichiarazione a meno che non sia spuntata la seguente casella:

the specification of which is attached hereto unless the following box is checked:

(...) il
è stata depositata una domanda di brevetto statunitense numero o una domanda di brevetto internazionale PCT numero
che è stata modificata il
(se del caso)

(x) was filed on **July 28, 2000**

as United States Application Number
or PCT International Application Number
PCT/IT00/00321 and was amended on
(if applicable)

Dichiaro inoltre con il presente di aver letto e compreso il contenuto della descrizione sopra indicata, comprese le rivendicazioni, come rettificata da qualsiasi emendamento a cui si sia accennato sopra.

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

Riconosco il mio dovere di rivelare informazioni che costituiscano materiale per l'esame della presente domanda secondo i termini del Titolo 37, Codice dei Regolamenti Federali, Comma 1,56(a)

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, § 1,56(a).

Italian Language Declaration

Con il presente rivendico i benefici di priorità per l'estero come stabilito dal Titolo 35, Codice degli Stati Uniti, Comma 119 per qualsiasi domanda di brevetto (o brevetti) straniera o per qualsiasi certificato di invenzione sotto elencato, ed ho anche elencato qui sotto tutte le domande di brevetto e certificati d'invenzione stranieri aventi una data di presentazione anteriore a quella della domanda per la quale si rivendica la precedenza.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior foreign applications Domande all'estero precedenti			Priority claimed Priorità rivendicata			
(Number) (Numero)	(Country) (Paese)	(Day,Month,Year Filed) (Giorno, Mese, Anno di Deposito				
MI99A001783	**ITALY**	**06 August 1999**	**(X)** Yes	(...) No	(...) Yes	(..) No
(Number) (Numero)	(Country) (Paese)	(Day,Month,Year Filed) (Giorno, Mese, Anno di Deposito)	**(X)** Yes	(...) No	(...) Yes	(...) No
MI2000A000306	**ITALY**	**28 July 2000**				
(Number) (Numero)	(Country) (Paese)	(Day,Month,Year Filed) (Giorno, Mese, Anno di Deposito)	(...) Yes	(...) No	(...) Yes	(...) No

Con il presente rivendico il beneficio previsto dal Titolo 35, Codice degli Stati Uniti, Comma 120, per qualsiasi domanda (o domande) di brevetto sotto indicate, ed entro i limiti nei quali il materiale indicato in ciascuna delle domande di brevetto non è stato rivelato nella precedente domanda di brevetto americana nel modo previsto dal primo paragrafo del titolo 35, Codice degli Stati Uniti, Comma 112, riconosco il mio dovere di rivelare il materiale d'informazione, così come viene definito nel titolo 37, Codice dei Regolamenti Federali, Comma 1,56(a), che possa essere venuto ad aggiungersi nel periodo intercorso tra la data di presentazione della domanda precedente e la data nazionale o internazionale da PCT di presentazione di questa domanda.

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1,56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.) Numero di domanda	(Filing Date) (Data di deposito)	(Stato Giuridico) (concessa, abbandonata)	pendente,	(Legal Status) (patented, abandoned)	pending,
(Application Serial No.) Numero di domanda	(Filing Date) (Data di deposito)	(Stato Giuridico) (concessa, abbandonata)	pendente,	(Legal Status) (patented, abandoned)	pending,

Dichiaro inoltre con il presente che tutte le informazioni da me fornite sono per quanto mi consta vere e che tutte le affermazioni da me fatte sono per quanto mi consta vere; dichiaro inoltre che quando ho fatto queste affermazioni ero al corrente del fatto che false dichiarazioni fatte intenzionalmente sono punibili con multa o incarcerazione o ambedue, secondo quanto stabilito dalla sezione 1001 del Titolo 18 del Codice degli Stati Uniti e che tali informazioni intenzionalmente false possono mettere a repentaglio la validità della domanda di brevetto rilasciata in base ad esse.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Italian Language Declaration

PROCURA: Io, sottoscritto inventore, nomino con la presente il seguente Procuratore (o Procuratori) o Agente (o Agenti) che si incarica di perseguire questa pratica e di portare a termine tutte le operazioni necessarie all'Ufficio Brevetti pertinenti a questa pratica. (Elencare il Nome e il Numero di Matricola)

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. (*List name and registration number)*

Recapito per la corrispondenza:

Send correspondence to:

Messrs.
Samuels, Gauthier & Stevens
225 Franklin Street, Suite 3300
Boston, Massachusetts 02110
U.S.A.

Telefonare a: (Nome e Numero)

Direct telephone calls to: (name and telephone number):

Nome completo dell'inventore primo o unico	Full name of sole or first\ inventor
CATTANEO Antonino	
Firma dell'inventore Data	Inventor's signature Date
1 Antonio Cattaneo	
Residenza	Residence
Via Beirut 2-4 – 34014 TRIESTE - ITALY IT X	
Cittadinanza	Citizenship
Italian	
Recapito o Casella Postale	Post Office Address
Via Beirut 2-4 – 34014 TRIESTE - ITALY	
Nome completo del secondo inventore, se esistente	Full name of second joint inventor, if applicable
2 **CAPSONI Simona**	
Firma dell'inventore Data:	Inventor's signature Date
Simona Capsoni	
Residenza	Residence
Via Beirut 2-4 – 34014 TRIESTE - ITALY IT X	
Cittadinanza	Citizenship
Italian	
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Nome completo del terzo inventore, se esistente	Full name of third joint inventor, if applicable
3 **RUBERTI Francesca**	
Firma dell'inventore Data:	Inventor's signature Date
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(Supply similar information and signature for third and subsequent joint inventors)

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